Extracellular adenosine impact on T cell reactivation and macrophage polarization under hypoxia

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Master Thesis

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Abstract

Glioblastoma (GBM) is the most frequent and aggressive primary malignant brain tumour for which research of efficient treatments is of paramount importance. Indeed, despite decades of research, the life expectancy of patients with GBM has not changed significantly with the use of standard treatments (tumour resection, chemotherapy, ionising radiations).

Solid tumours share common features which include the presence of hypoxic regions surrounding the tumour core. Due to the lack of nutrients, a lot of cells die in these regions and release various compounds such as adenosine (ADO) into the extracellular matrix. Adenosine signalling properties under normal physiological conditions have been widely studied. However, in the context of cancer, even though it was proposed that ADO attenuates the immune response, it is still unclear how high extracellular ADO levels impacts the responsiveness of immune cells. Nevertheless, evidence suggests that extracellular ADO functions are mostly immunosuppressive and limit excessive inflammation.

T cells and macrophages are two main actors of antitumour immunity. In this study we explored the effects of extracellular ADO addition and oxygen deprivation in the macrophage polarization process through the analysis of surface markers (CD206, CD68, CD80, CD86), gene expression (*Nos2, Stat1, Il-1b, E-Cad, Arg1, Mrc1*), and cAMP generation. We also explored how T cell responses after reactivation were affected by extracellular ADO and hypoxia and whether or not inhibition of A_{2A} receptor (A2ar) could counterbalance these effects. To assess this, we analysed expression of T cell surface markers associated with activation and exhaustion (CD69, CD39, PD1) as well as IFN-γ secretion and cAMP levels.

Contrary to what was often proposed, we observed a mostly beneficial effect of extracellular ADO and hypoxia in regards to the M1/M2 macrophage balance, based on the markers that we used. Our results also confirmed that CD8 effector functions were impaired by high doses of extracellular ADO. Nevertheless, ADO immunosuppressive effects were partially counterbalanced by the use of an A2ar inhibitor. Finally, our data suggest that adenosine deaminase (ADA) inhibition could be a possible way to restore antitumour immunity if combined with A2ar inhibition.

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Abbreviations

A2ar:	A_{2A} Receptor
A2br:	A_{2B} Receptor
APC :	Antigen Presenting Cell
ADA:	Adenosine Deaminase
ADO :	Adenosine
ADOi :	Adenosine Inhibitor
ADP :	Adenosine Diphosphate
AMP :	Adenosine Monophosphate
ATP :	Adenosine Triphosphate
BMDM :	Bone Marrow-Derived Macrophages
cAMP :	Cyclic Adenosine Monophosphate
CD:	Cluster of Differentiation
CTL:	Cytotoxic T cell
DC:	Dendritic Cell
ELISA :	Enzyme-Linked Immunosorbent Assay
HLA:	Human Leukocyte Antigen
IFN-γ:	Interferon γ
IL-2/4/10/13:	Interleukin-2/4/10/13
M-CSF:	Macrophage-Colony Stimulating Factor
MHC :	Major Histocompatibility Complex
NK:	Natural Killer Cell
OVA:	Ovalbumin
PAMP:	Pathogen Associated Molecular Pattern
Pento:	Pentostatin
PRR:	Pattern Recognition Receptor
qPCR :	Quantitative Polymerase Chain reaction
TAA:	Tumour Associated Antigen

TCR :	T cell Receptor
TGF- α/β:	Transforming Growth Factor α/β
TIL:	Tumour Infiltrating Lymphocyte
TLR:	Toll-Like Receptor
TME:	Tumour Microenvironment
TNF-α:	Tumour Necrosis Factor α
TSA:	Tumour Specific Antigen
VEGF :	Vascular Endothelial Growth Factor

1 Introduction

1.1 Overview of the immune system and its main cells

The immune system can be described as a network of cells driven by biochemical processes in order to maintain homeostasis in the body, it fights whatever may disturb this homeostasis. This includes bacteria, virus, parasites and many other pathogens. It includes cancer as well.

There is a wide variety of immune cells with very specific roles, however they are all derived from a same progenitor (as well as erythrocytes and platelets): the haematopoietic stem cells from the bone marrow.[1] Two differentiation pathways can be described and will give rise to all the immune cells. The myeloid progenitor cells will finally give rise to cells which includes macrophages, polymorphonuclear neutrophils (PMN), dendritic cells (DCs), basophils, eosinophils, erythrocytes and platelets. On other hand, the lymphoid progenitor cells will differentiates into T cells, B cells and Natural-Killer cells (NKs).

Two types of response from the immune system can be considered. The first one to act, the innate response, takes place very rapidly after the pathogen entered the body (minutes/hours). Innate response is not specific to individual antigens but recognizes precise conserved patterns. This response is mediated by the myeloid cells and is considered as the first immune barrier of the body that acts at different levels. The complement system, for example, is a plasma protein system that marks and opsonizes pathogens. There is also the physicochemical barrier furnished by the skin that protect us against most intrusions.[2] As mentioned, innate response also relies on the recognition of conserved features among pathogens such as pathogen associated molecular patterns (PAMPs) by their pattern recognition receptors (PRRs). This recognition leads to a downstream secretion of cytokines, chemokines and type-I interferons, therefore infection and will initiate the adaptive response.[3]

The other type of immune response is called the adaptive response. This response is highly specific to a given antigen and offers a high capacity of adaptation but starts after the innate response (days). The cells involved in this response are called lymphocytes and are divided in two mains cell types: T and B cells. Adaptive response is based on an antigen recognition principle. Antigens are molecules or molecular structures of different types (peptides, proteins, lipids, polysaccharides, nucleic) that can be self (self-structures) or non-self (foreign material). When recognised as non-self by the immune system an adaptive lymphocytes response is triggered. These antigens can either be surface antigens or soluble antigens. As mentioned, the adaptive response is antigen-specific, it means that the T cells with the right TCR (T cell receptor) must encounter and recognize its specific antigen in order to initiate an enormous clonal expansion that will lead to a lymphocyte response. Resolution happens with the death of the pathogen and the generation of

memory cells that will be maintained for years and will eventually reactivate and trigger the response faster if the same pathogen is detected again.

In the context of cancer, the cells are prone to frequent mutations because of the high rate of uncontrolled divisions they produce modified proteins that are recognized as non-self and therefore triggers a similar reaction. These proteins are called tumour-specific antigen (TSA) and are present only on tumour. Oppositely, tumour-associated antigen (TAA) are only present in high quantity in tumour (and in low quantities in healthy cells), they are self but are aberrant in terms of site of expression or density.[4,5]

Although the two immune responses are temporally different and involve different cell types, they are not exclusive, on the contrary, they are codependent and work in concert.

T cells, macrophages and DCs are the main cells playing a role in antitumour immunity. The focus for the rest of the thesis will then be put on the role of these cells, particularly T cells and macrophages, in the context of GBM.

1.2 The Major Histocompatibility Complex (MHC) and Antigen Presenting Cells (APCs)

In order to recognize an antigen they are specific for, T cells need this antigen to be presented to their TCR. This is mediated by a membrane polypeptide complex called MHC (H-2 complex in mouse and HLA, for Human Leukocyte Antigen, in Human), which is present in every nucleated cell.

MHC has two roles and will therefore be divided in two classes. The first goal of MHC will be to act as a mirror of the cell cytosolic content by presenting fragments of cytosolic peptides through MHC class I molecules. MHC class I molecules will be monitored by the immune system ($CD8^+$ T cells and NK cells)[6] and eventually leads to cell death trough degranulation of perforin/granzymes if the presented antigen is recognized as non-self. This is the role of the MHC class I which, as mentioned earlier, is present in all nucleated cells. Note that the degranulation process will be detailed in the $CD8^+$ section.

On the other hand, MHC will also allow to monitor what is in the extracellular content[7], this will be mediated by the MHC class II. Contrary to MHC class I, class II will only be present in certain cell types called APCs. The particularity of APCs is that they are able to phagocyte (ingest) entities of the extracellular content (including cell fragments, bacteria and other particles). They are being digested into acidic endocytosis vesicles, then a part of these particles is associated with an MHC class II and goes into the cell surface to be expressed. As with class I MHC, class II MHC will be monitored by lymphocytes, but unlike the latter, they will be recognized by $CD4^+$ lymphocytes and this recognition will not lead to cell death but will turn the naive $CD4^+$ into either an effector that will downstream activates $CD8^+$ T cells or a memory T cell. The APC family includes: dendritic cells, macrophages and B cells.[8]

1.3 T cell maturation, activation and differentiation

1.3.1 Maturation

As mentioned earlier, T cells are derived from haematopoietic stem cells in the bone marrow, however, contrary to the other cells that keep maturing in the bone marrow, T cell precursors migrate to the thymus where they will be subject to a strict process of selection. As stated, T cells have to recognize antigens through their TCR: their receptor has to be specific for this antigen and only this antigen. The diversity is obtained by an assemblage of the four TCR gene segments through a V(D)J domains recombination.[9] The newly obtained TCRs undergo a double quality control test called positive selection and negative selection. Only the T cells that recognize self-MHC class I or II will survive (positive selection), then these that binds too strongly to self-antigens presented by APCs are eliminated, in order to avoid autoreactive T cells (negative selection). When they enter the thymus, T cells are double negative ($CD8^-/CD4^-$), during the selection process they becomes double positive ($CD8^+/CD4^+$) and at the end they are single positive ($CD8^+$ or $CD4^+$) based on which MHC they recognised and will bind to. This MHC restriction forces CD8 T cells to only interact with MHC class I while CD4 T cells can only interact with MHC class II.

At this step T cells are still considered immature (naive) T cells. They will travel to the secondary lymphoid tissues (spleen, lymph nodes, GALT) and will interact with different cells, including APCs and other lymphocytes. T cells will eventually encounter the antigen they are specific for and activate, proliferate and differentiate, giving rise to a lot of subtypes with different roles.

1.3.2 Activation and costimulation

In order to get activated the TCR-antigen recognition (signal 1) alone is not sufficient, T cells need costimulation (signal 2) as well otherwise they become anergic, their proliferation and cytokine secretion are therefore inhibited. The costimulation (also referred as “danger signal” in literature) requires the interaction of many T lymphocyte membrane proteins with their ligands, present on APC membranes.[10] The main one is CD28 (T cells) and its ligand is CD80/CD86 (APCs), but there are others like ICOS, 4-1BB, OX40 or CD27.[10,11]

Beside costimulatory molecules, there are co-inhibitory molecules that will compete as well for the same sites (CTLA4 for CD80 or PD1 for PDL1 for example)[12] in order to control the T cells activation, however in context of tumour and inflammation, these co-inhibitory accumulates, leading to a reduction of effector T cell generation.

As mentioned, costimulatory signal is mandatory for both T cell proliferation, survival and to let them trigger their effector functions, however another signal, the cytokine signal (signal 3), plays a very important role too. Recent studies suggest that signal 3 plays a role in CD8 T cells expansion and differentiation through type I IFN (α, β) and IL-12. Literature

says that they act as a switch that determines whether TCR-MHC interaction and costimulation will lead to a strong response through effector functions (presence of inflammatory cytokines/signal 3) or through tolerance and anergy (absence of inflammatory cytokines and signal 2).[13]

1.3.3 Differentiation, subtypes and roles

As mentioned, T cells become either $CD4^+$ either $CD8^+$ at the end of the double selection process. Although this is a first step in the differentiation cascade, there are a lot more subtypes into which T cells will eventually differentiate, each one with a specific role and a tightly regulated differentiation process.

$CD4^+$ T cells play a major role in the establishment of the adaptive response as well as its maintenance and will therefore differentiate into many different cell types. Among these cells there are regulatory T cells (Treg), mandatory in order to maintain homeostasis and peripheral tolerance through immunosuppressive functions on effector T cells, thence preventing autoimmune response. There are also T helpers (Th1, Th2, Th17 and Tfh are the main ones) that will coordinate and stimulate the T effector response through cytokine secretion that will help them proliferate and differentiate, each Th subtype being specialized against one type of pathogen and will activate different immune cell types.

Some T cells will eventually differentiate into memory T cells that will ensure a fast and effective response toward an already met antigen (TEM for effector memory in the peripheral tissues and TCM for central memory, those located mostly on lymphoid tissues).[14] They can be either $CD4^+$ either $CD8^+$ memory T cells.

Finally $CD8^+$ T cells, that turned from naive to effector after they received the 3 signals, are the cells that will mediate the main effector response through cytotoxicity mediated by MHC-peptide recognition. They will monitor the other cells MHC and if they encounter the antigen they are specific for they will degranulate vesicles containing perforin and granzyme. Perforin will assemble on the target cell surface to form a tunnel directly inside the cell where granzyme can go to trigger the cell death program (apoptosis) through a caspase cascade, leading to the cell death.[15]

Because $CD8^+$ T cells are one of the main actors in the antitumoural response, they are a key target for immunotherapies that try to improve their effectiveness (limited by tumour immunosuppressive mechanisms), as well as their poor infiltration into the tumour site which is a big issue in the context of cancer.[16] $CD8^+$ T cells will therefore be a major focus in our experiments.

1.4 Macrophage polarization

Macrophages, as mentioned, are APCs that act both with the innate response through PAMPs recognition and with the complement, but as well trigger the adaptative response through antigen presentation.

In response to chemical signals (chemokines), leukocytes will be attracted and slowed by many interactions between proteins from the leukocyte and the endothelium surfaces (selectins, integrins, CD34, SLe^x)[17] to finally penetrate the inflamed tissue by passing thought two endothelial cells. This phenomenon is called diapedesis and all leukocytes resort to it to infiltrate tissues. Monocytes are circulating cells but will differentiate into macrophage once they penetrated a tissue. Macrophages are given different names based on their tissue localisation, there are for example microglia (brain), osteoclast (bones), alveolar macrophages (lungs), etc.[18]

Macrophages, once in the inflamed/tumour site, can polarize into two different populations, M1 (also referred to as "classically activated macrophages") and M2 (also referred to as "alternatively activated macrophages"). These two populations arise from different pathways, have different functions and express different markers, however they are not a static or binary state. Defining a single molecular marker signature was therefore proven to be challenging.[19]

M1 macrophages are considered pro-inflammatory. They polarize in response to simultaneous high levels of inflammatory cytokines (secreted by Th1) such as IFN-γ or TNF-α and stimulation coming from microbial factors like LPS (Lipopolysaccharide).[20] They will respond by secreting pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1, IL-12, etc.) and will represent an important source of reactive oxygen species (ROS) and nitrogen radicals, therefore granting them improved microbicidal properties, antitumour defence as well as for tissue destruction.

On the other hand, M2 macrophages have anti-inflammatory properties. Indeed, chronic inflammation induces tissue destruction and is not an ideal state for the tissue in long term. M2 macrophages are here to balance the M1 macrophages effect. They are called "alternatively activated macrophages" because contrary to M1 macrophages that will be polarized by Th1 secretions, M2 macrophages will be polarized by Th2 secretions such as IL-4 and IL-13. They will induce tissue reparation and resolution of inflammation through anti-inflammatory secretions, trophic factor synthesis (proangiogenic and remodelling) and high endocytotic clearance.[20,21]

Due to their properties M2 macrophages also play a role in the tumour growth and progression while, in contrast, M1 macrophages delay this progression. These cell populations and the way they are shifting toward a type or the other will therefore be a major interest in this thesis.

1.5 Glioblastoma and current clinical management

Among all cancers and more generally all solid tumours, GBM may be one of the most aggressive and malignant, despite all the recent advances in the research field of oncology. Gliomas are brain solid tumours derived from macroglial cells such as astrocytes, oligodendrocytes, ependymocytes. Glioblastoma is a subtype, the most malignant and frequent brain tumour (60 % of the malignant brain tumours) derived from astrocytes.[22] GBMs are divided in two other subtypes, based on their clinical presentation. The majority of them are primary GBMs (90-95%)[23] and are *de novo*, meaning that they are not arising from the progression of a lower grade astrocytoma. Secondary GBMs on other hand are typically the evolution from a lower-grade astrocytoma that became worse and worse over time.[24] Both types are impossible to discriminate morphologically and share the same terrible outcome.

Under normal circumstances, glial cells play many essential roles within the brain environment. Astrocytes, for example, ensure the maintenance of the blood brain barrier (BBB) function as well as their role in the ionic homeostasis or synaptic transmission. Oligodendrocytes, on the other hand, are myelin producing cells, whose myelin acts as an electric isolation on the axon, granting them a super-fast transmission capacity, known as saltatory transmission. Ependymal cells are another type of glial cells, whose main role is to secrete cerebrospinal fluid (CFS). Finally, microglia corresponds to the resident macrophages of the brain. They act as warden and are the main central nervous system (CNS) immune defence.[25,26]

In the context of glioma however, macroglial cells cannot fill their role anymore. They are dysregulated, do not respond to the usual signals and start to divide in an uncontrollable way, leading to many subsequent complications.

Despite decades of research, the life expectancy of patients with GBM has not changed significantly and people at 2, 3, 4 and 5 years after a combined treatment of both ionising radiations and chemotherapeutic agent temozolomide[27] still suffer a very low survival rate of respectively 27.2 %, 16 %, 12.1 % and 9.8 %.[28,29]

Even after an aggressive tumour resection coupled with chemotherapy and IR, 90 % of the treated patient ended up with the tumour recurring at its original site leading to a mean 12-14 months survival post-diagnosis.[28]

Gliomas are usually classified based on their histological criteria (grade I-IV gliomas), GBM being the highest grade (IV). This grade was used historically and is still a good predictor of the outcome, however recent progress in the field has permit to distinguish different expression profiles[30] as well as distinct DNA methylation[19,31] on grade IV glioma. The tumour profiling tends to be a better predictor of the clinical outcome than the histological criteria alone and could be used as an interesting tool for the development of future therapies.

1.6 GBM aggressiveness, plasticity and immunosuppressive properties

Different reasons can explain this low survival rate. First comes the location. The tumour being in the brain makes it obviously harder to track and treat. On the other hand, as stated, there are the different expression profiles and DNA methylation between GBMs. This inter-patient tumour heterogeneity plays a major role as well because a given treatment will not have the same effect on two patients. Furthermore, cancer cells from the same patient show a wide diversity as well, in terms of phenotypes, morphology, gene expression, motility and more important, in terms of potential (invasive, proliferative and immunogenic).[32] This intratumoural heterogeneity makes tumour obviously harder to treat because cells will not react the same way to a given treatment which impairs therapy response.[33] Another feature concerns the infiltration of immune cells within GBM tumour site, which is low and encompasses exhausted T cells that express checkpoints molecules (i.e. PD-1, LAG3) and are therefore inefficient.[34] This feature is strongly associated with the prognosis and the responsiveness to immunotherapies.[35,36] Finally, there are not many immune cells in the brain, this phenomenon called the immune privilege also occurs in the eyes, testes, placenta and foetus to protect these tissues against inflammation.[37] It gives a fertile ground for the tumour to proliferate.

1.7 Tumour microenvironment and hypoxia

The National Cancer Institute (NCI) says that tumour microenvironment is described as the surrounding of the tumour, which include healthy cells, immune cells, blood vessels and molecules.[38]

The tumour microenvironment (TME) is a major regulator of tumour development. In this microenvironment the immune cells that infiltrates exert a poor immune response against cancer cells that escapes the classical immunosurveillance through different mechanisms that includes: the recruitment of immunosuppressive cells to produce an immunosuppressive microenvironment (IL-10, TGF-β). The cancer immunoediting that implies that cancer clones evolve in order to avoid elimination by leukocytes (loss of immunogenicity/antigenicity).[39] This whole phenomenon is called immune escape and facilitates tumour progression.

As the cancer progresses, the cancer core requires more oxygen and glucose supply and is doing it through VEGF (vascular endothelium growth factor) secretion, a signalling protein involved in the formation of new blood vessels.[40] The blood supply is driven toward the core, however, the poor blood vessel formation, coupled with uncontrolled fast divisions makes the microenvironment hypoxic and eventually necrotic. These regions are a common feature shared within all solid tumours, which includes GBM.[41,42] The necrotic zone represents the tumour core and is surrounded by the hypoxic (perinecrotic) zone. A

region where cancer cells keep dividing, overlaying, leading to a local oxygen deprivation (***Fig.1***). Because of these hostile conditions, the cells die and release different signalling components, including extracellular adenosine triphosphate (ATP). The TME eventually also turns acidic due to the high level of aerobic glycolysis. Indeed, cancer cells preferentially use glycolysis over oxidative phosphorylation, even when there is enough oxygen for the oxidative phosphorylation which would be more profitable in terms of ATP generation (Warbug effect).[43,44]

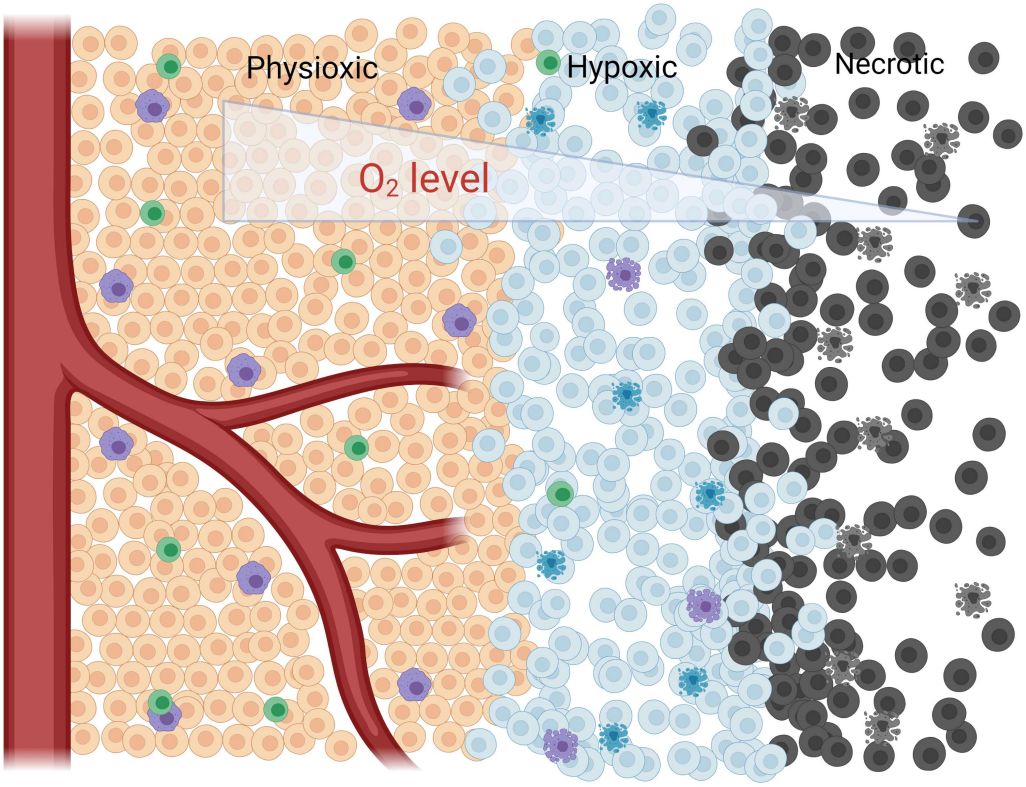


Figure 1: Representation of a solid cancer regions. *Physioxic area (yellow), hypoxic area (blue) and necrotic core (black) with immune cells within them (violet: macrophages, green: T cells). Note that this figure is a representation. In vivo, there is not a sharp separation between the regions and the O_2 level looks more like a gradient, as for the cell shape. Only one hypoxic area is represented in this figure, however, in vivo there might be multiple hypoxic and necrotic areas, depending on the oxygen supply and the level of cell division.*

The optimal oxygen saturation is not the same between tissues; this is why the term "physioxic" is more suited than "normoxic" when referring to a normal oxygenation level. For instance, in secondary lymphoid tissues a physiologic oxygen saturation is about 2.5% O_2[45] and the same goes for most human tissues whose physioxic oxygen saturation ranges from 1% to 11%, with a maximum saturation of 14.5% in pulmonary alveoli.[41,46,47]

For the following when mentioning hypoxic condition, it will refer to a 1% oxygen gas mix and when we refer to a normoxic condition it will refer to a 21% oxygen gas mix

(atmospheric level), which in reality would be an hyperoxic condition, but has been proven to be a better condition for cells culture and is classically used. Note that the oxygen saturation in the culture media is lower than the gas mix.

Hypoxia is described in literature as a powerful mediator of immunosuppression that limits the efficiency of antitumour immunity through direct effects and indirect effects which includes the release of extracellular ADO.[48]

1.8 Adenosine cascade, receptors and cellular effects

In normal physiological conditions, ATP molecules play an essential role, that powers up all the energy-dependent processes in the cells. ATP can be converted by different enzymes into its derivatives, acting as signalling molecules: adenosine diphosphate (ADP), adenosine monophosphate (AMP) and ADO (***Fig.2***).[49]

These derivatives include ADO, a molecule known to be one of the four nucleotides that forms deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

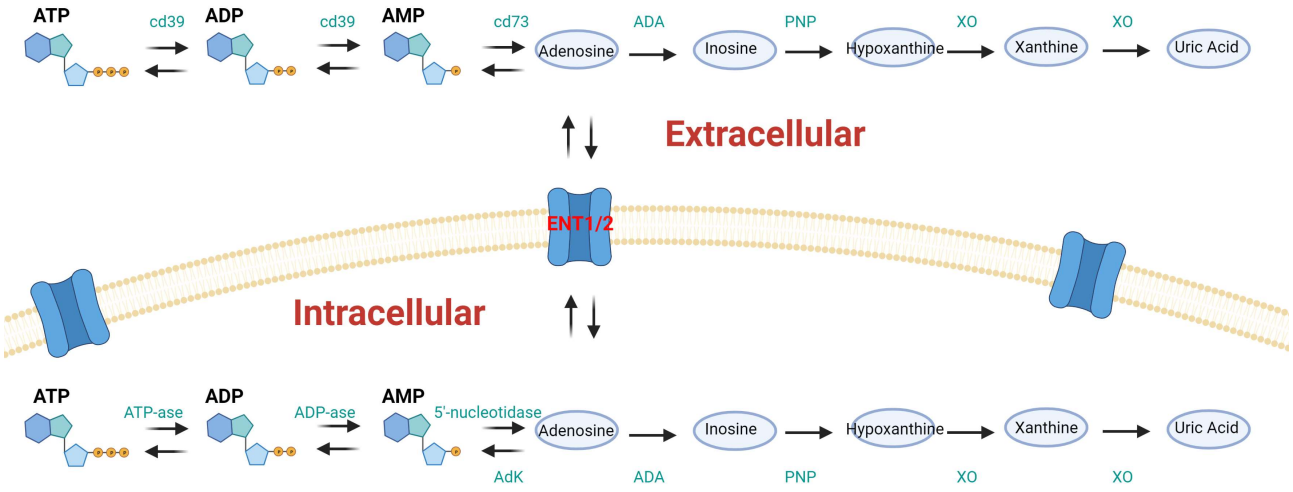


Figure 2: Summary of the ADO cascade and its main actors. *The transformation from ATP to its derivative takes place both extracellularly, intracellularly and inside the nucleus. Most of the enzymes are expressed on the cell surface only (CD39, CD73), but some can either be expressed on the cell surface either be secreted on the close extracellular content such as ADA and Purine nucleoside phosphorylase (PNP). ADO can circulate both from intra- to extra-cellular and from extra- to intra-cellular through the Equilibrative nucleoside transporter (ENT1/ENT2), therefore modify this equilibrium.*

Adenosine is present constitutively at all cells levels, but its concentration is low in the extracellular content under normal conditions.[50] Under stressful conditions however, this concentration can increase substantially, these conditions include hypoxia. Indeed, as shown in ***Fig.1***, in context of a low oxygen tension, there is tissue inflammation, cells die and release extracellular ATP which will subsequently form ADO and inhibit this inflammation, therefore the immune response.[51,52]

Extracellular ADO can bind to different receptors (***Fig.3***). To date, four of them were described: A1r (ADORA1), A2ar (ADORA2A), A2br (ADORA2B), and A3r (ADORA3). They are all classified as G protein-coupled receptor (GPCRs), meaning that once the receptors bounds their ligand (ADO) they will change their conformation, starting a signal transduction inside the cell that will involve a G protein as an intermediary messenger.[53] All these receptors are crossing the lipid bilayer membrane seven times, granting them also the name of 7-Transmembrane receptors (7TMr).[54]

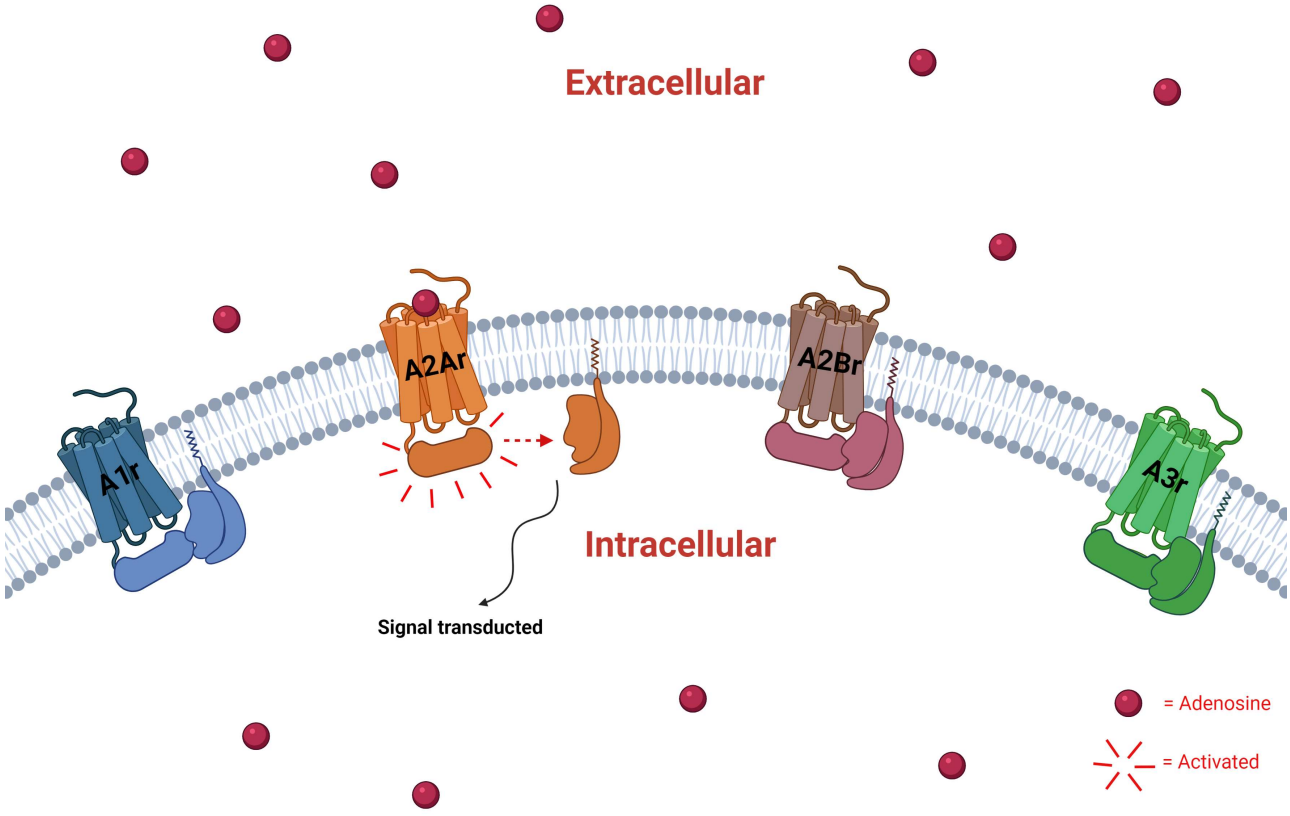


Figure 3: The four ADO receptors and their activation mechanism. *Extracellular ADO binds to an A2ar transmembrane domain, initiating a conformational change. The trimeric G-protein is activated (α domain), β and γ subunits moves along the membrane to signal and induce a cellular response downstream. Note that on this figure only A2ar was shown activated, however, all of them can bind ADO the same way (with different affinity) and create an intracellular response.*

The main differences between ADO receptors are the tissue and cell type they are located on as well as their sensitivity. Recent study showed that A2a and A2b receptors are the most expressed in human brain tumours.[55] A2ar was shown to be mostly expressed on the T cell surface[56,57] while A2br seems to be ubiquitous with a higher expression on APCs.[58,59] It was shown that A2ar has a higher affinity for ADO than A2br as it can get activated from nanomolar concentrations of ADO while A2b requires micromolar concentrations.[60]

Adenosine signalling properties in healthy tissue have been widely studied. However, in cancer, even though it was proposed that ADO attenuates the immune response, it is still unclear how high extracellular ADO levels impact the responsiveness of immune cells. Evidences suggest that extracellular ADO functions are mostly immunosuppressive and act in order to avoid excessive inflammation. Signal transduction by ADO will lead to secretion of anti-inflammatory cytokines as well as downregulating the secretion of molecules involved in the immune infiltration (chemokines).[61,62]

As mentioned earlier, in $CD8^+$ T cells, this anti-inflammatory cocktail induces an exhausted phenotype with poorly activated $CD8^+$ T cells that express high levels of immune checkpoint molecules (i.e. PD1) and the ectonucleotidase CD39 (to convert ATP to ADO). Exhausted $CD8^+$ also have a low expression of activation markers (e.g. CD69) resulting in failure to produce effector response through cytokine secretion (IFN-γ, TNF-α) or cytotoxic molecules such as granzymes and perforin.[62–64]

In macrophages, the anti-inflammatory microenvironment induced by ADO is shifting the balance toward an M2-like phenotype which imply the expression of different surface marker. Well established M2 mouse and human markers from literature include CD206 (mannose receptor 1, coded by *Mrc1*), CD68 and CD163; while the main markers used for M1 macrophages are CD80 and CD86 that interact with CD28 during the costimulation of T lymphocytes. There are others M1 markers (e.g. CD64 and CD32) but CD80 and CD86 are the most used in literature.[20,65–67] Two macrophages subtypes imply different functions and two different expression patterns with different gene signatures, specific to each subtype. M2 macrophages will for example secrete immunosuppressive cytokines (e.g. IL-10), while M1 macrophages will typically secrete pro-inflammatory secretions that will maintain an immune response (e.g. TNF-α).

1.9 Aims of the thesis

Due to ADO immunosuppressive properties, targetting its extracellular receptors on immune cells might be a potent solution in order to restore their antitumour function. Furthermore, ADO effects and dynamic at high concentrations in the context of hypoxia still remain unclear and need to be explored. The experiments will therefore be focused in three main axes.

First and foremost, the first goal will be to measure and quantify the cancer heterogeneity. This will be done in order to illustrate how much the heterogeneity is an important feature of cancer that can heavily affect and narrow a response to treatment. To illustrate this feature, expression of different metabolism genes and hypoxia associated genes (A_2*A, Ca9, CD39, CD73, H2s, Slc2i*) will be measured on human GBM samples as well as the difference of VEGF secretion of four cancer cell lines in response to hypoxia.

The second goal will be to measure the impact of extracellular ADO addition in the macrophage M1/M2 balance in the context of normoxia and hypoxia. Then to try to push them toward a pro-inflammatory (M1) phenotype instead the anti-inflammatory M2 phenotype through the addition of A2ar and A2br inhibitors (LAS101057). To measure the effect of inhibitor on the balance shift, macrophage surface markers (CD206, CD68, CD80, CD86), gene expression (*Nos2*, *Stat1*, *Il-1b*, *E-Cad*, *Arg1*, *Mrc1*) and cAMP generation will be analysed.

Finally, the third goal will be to monitor the $CD8^+$ T cell reactivation and response in presence of different ADO concentration and see if ADO immunosuppressive properties can be counterbalanced by the addition of A2ar inhibitor (Istradefylline). To measure the T cell response, surface markers of activation and exhaustion (CD69, CD39, PD1) will be examined as well as IFN-γ secretion and cAMP levels.

2 Material and methods

Material

2.1 Mice

All the results of the mice experiments were issued from mice colonies bred and housed in the CMU animal facility. Every experiment was done in accordance to the Swiss regulation (SCAV).

C57BL/6

C57BL/6 mice are the most popular laboratory rodent for immunology studies. They are an inbred strain and were initially developed a century ago at the Bussey Institute for Research in applied Biology. Despite being refractory to many tumours, this strain possesses a permissive background, allowing the expression of many mutations.[68]

OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/Crl)

OT-I mice are a transgenic mouse line that express a T cell receptor (TCR) specific for chicken Ovalbumin$_{257-264}$ (Ova). The receptor is composed of α-chain variable region 2 ($V_\alpha 2$) and β-chain variable region 5 ($V_\beta 5$), these chains are pairing with the CD8 co-receptor.[69] Adding Ova in the presence of these cells will start a T cell response and are therefore an excellent and widely used tool in immunology.

2.2 Cancer cell lines

Jurkat cells

Jurkat cells are human T cells widely used in the field of immunology. They were established in 1977 from the peripheral blood of a young boy who had acute lymphoblastic leukemia.[70] They are a good model that will be used in order to collect data of the ADO effect from human cancer cell line.

Raw cells

Raw cells are mouse monocytes/macrophages-like cells from the murine RAW 264.7 line. The line is derived from BALBC/c mice (a laboratory-bred, albino and immunodeficient line)[71] transformed with Abelson-leukemia virus. They are capable of pinocytosis,

phagocytosis and antibody-dependant cytotoxicity.[72] This model will give us information about the ADO effects on a macrophage cancer cell line.

2.3 Human GBM samples

Tumour samples from HUG patients with high-grade gliomas were removed and frozen after a tumour resection. They were kindly provided by Dutoit-Migliorini's lab for expression analysis.

2.4 Incubators

During generation of the cells, T cells and Jurkat cells were cultured at 37°C with 21% O_2 and standard 5% CO_2 for 7 days. Macrophages and Raw cells were incubated at 37°C with 21% O_2 and standard 8% CO_2 for 7 days.

2.5 Hypoxic Chamber

For all experiments requiring and hypoxic environment, cells were cultured at 1% O_2 in our hypoxic station (Ruskinn InVivo$_2$ 300) after complete generation of the cells during reactivation (7 days) for the time of treatment. The presets were always 37°C, 8% CO_2, 1% O_2.

Methods

2.6 Cell acquisitions and cultures

2.6.1 T cells

T cells were obtain from OT-I mice spleen. After being sacrificed by CO_2 asphyxia, mice got their spleen removed and crushed within a 7 nM filter. Red blood cells were removed by adding ACK (Amonium Chloride Potassium) lysing buffer (Gibco) for 2 minutes. Cells were then plated into 6-well plates (8 million cells/well) and were grown with Dulbecco modified Eagle medium (DMEM) (Gibco) supplemented with 1% HEPES (Gibco), 7% foetal bovine serum (FBS), 0.1% β-mercaptoethanol (BME, Gibco), 1% PenStrep mix (Gibco, 10.000u/mL Penicilin, 10.000µg/mL Streptomycin), 1% Glutamine solution (Gibco), 1% L-Arginine solution (1.16g/100mL, Gibco), 1% Asparagine solution (360mg/100mL, Gibco). Cells were incubated at 37°C, with 21% O_2 and 8% CO_2 for the 7 following days (***Fig.4***).

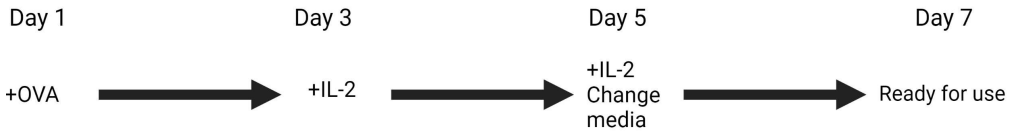


Fig.4: T cell generation Diagram. *Ovalbumin peptide was added Day 1 at a concentration of 10µM. On day 3, Interleukin-2 (IL-2) was added (100u/mL). On day 5, media was changed and IL-2 was added again (100u/mL), cells were split if confluence was too high.*

2.6.2 Bone Marrow Derived Macrophages

Bone Marrow Derived Macrophages (BMDM) were obtained from C57BL/6 mice bone marrow. After being sacrificed by CO_2 asphyxia, mice tibias and femurs were collected. Both extremity of the bones were cut and phosphate-buffered saline (PBS) was injected through the bones with a needle to collect the bone marrow. Cells were isolated with centrifugation and red blood cells were removed by adding ACK lysing buffer (Gibco) for 2 minutes. Cells were then plated into dishes (3 millions cells/dish) filled with 10 mL of RPMI Medium 1640, supplemented with 1% HEPES (Gibco), 10% FBS, 1% Sodium Pyruvate (NaPyk), 0.1% β-mercaptoethanol, 1% MEM Non-Essential Amino Acids Solution (ThermoFisher), 1% PenStrep mix (Gibco, 10.000u/mL Penicilin, 10.000µg/mL Streptomycin). Macrophages were split in three groups that received different treatments (***Fig.5***) in order to obtain M0, M1 and M2 polarized macrophages. They were incubated at 37°C with 21% O_2, 5% CO_2 for the 7 following days.

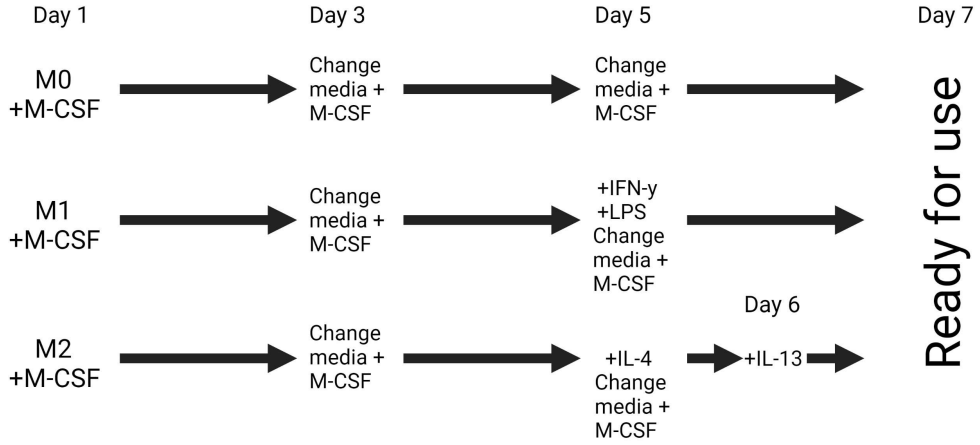


Figure.5: Macrophage polarization diagram. M0 *were plated with 10ng/ml Macrophage colony-stimulating factor(M-CSF) for 7 days starting day 1.* ***M1*** *were plated with 5ng/mL M-CSF for 7 days starting day 1. On day 5, Gamma Interferon (IFN-y) was added at a concentration of 20ng/mL, followed one hour later by lipopolysaccharide (LPS) at 100ng/mL.* ***M2*** *were plated with 25ng/mL M-CSF for 7 days starting day 1. On day 5, 20ng/mL Interleukin-4 (IL-4) was added, followed by 20ng/mL Interleukin-13 (IL-13) on day 6. Note that for the three macrophages types, media was changed on day 3 and 5 (and new M-CSF was added) in order to get rid of non-adherent cells.*

2.6.3 Jurkat cells

Frozen Jurkat cells were kindly provided by Dutoit-Migliorini's lab. They were thawed, then cultured in flasks with the same DMEM medium used for T cells cultures. They were passed once before use.

2.6.4 Raw cells

Frozen Raw cells were kindly provided by Dutoit-Migliorini's lab. They were thawed, then cultured in flasks with the same RPMI medium used for macrophages cultures. They were passed once before use.

2.7 Reactivation and treatment of T cells

After 7 days of culture, T cells were reactivated either with M0 cultured macrophages presenting OVA (M0 were incubated with 10µM OVA for 30 minutes in order to get the peptide present on their MHC) with a 1:3 ratio, either with Beads (Gibco by Thermo Fisher Scientific, Dynabeads^{tm}, T-Activator CD3-CD8) with a ratio of 1:1, either were not reactivated (control). Drugs were then added following different combinations (***Fig.6***). They were finally incubated in normoxic or hypoxic environment for 3 days before being analysed. We chose to monitor the T cell response to treatment after 3 days based on previous titrations. This time point seems to be the best in order to have the most products accumulation before T cells starts dying. We decided to monitor the macrophages-only response to treatment after 3 days as well with the same doses in order to match the response of reactivator macrophages from the T cell panel.

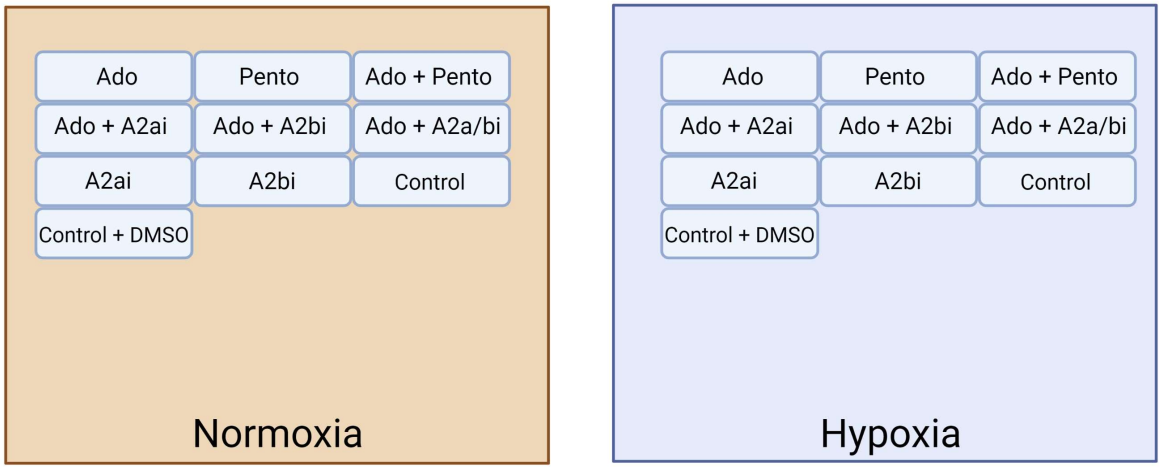


Figure 6: Table of treatments. *At Day 7, reactivated T cells and polarized macrophages were treated with different drug combinations at these concentrations: Adenosine (ADO) 0.5 mM and 1mM, Pentostatin (Pento) 50µM, Adenosine A2a receptor Inhibitor (A2ai, Istradefylline) 2µM, Adenosine A2b receptor inhibitor (A2bi, LAS101057) 5µM. All treatments stocks are frozen in DMSO, a vehicle control was therefore done at a concentration equal to the highest DMSO level condition (ADO+A2ai+A2bi, 1.2% DMSO). No significant difference was recorded between control and vehicle control in terms of cell viability or expression. All ADO, Pento, A2ai and A2bi were purchased from MedChemExpress (MCE).*

2.8 Assessment of cell viability

Cell viability was verified after extraction (D0) and after generation/reactivation (D7) with Trypan blue. This dye enters dead cells damaged membranes and stain them giving them a dark-blue colour under the microscope. In an opposite way, healthy live cells have impermeable membrane, dye cannot pass through and do not stain them, they appears as white under microscope.

A fraction of cells was mixed with Trypan blue and counted in a NeuBauer chamber in order to control the evolution of cell viability throughout the cell generation as a quality-control test.

2.9 Flow cytometry

Flow cytometry experiments were done on Gallios flow cytometer (Beckman Coulter) after 24 to 72 hours of treatment. For detailed flow cytometry procedures and principle, refer to section 3.1. Find below the detailed tables of antibodies used for each experiment with their final concentrations.

Antibody anti-	Host / Isotype	Clone	Fluorochrome	Supplier	Dilution
CD8	Rat IgG2a, κ	53-6.7	APC	Biolegend	1/500
CD279 (PD1)	Rat IgG2a, κ	29F.1A12	Pe-Cyanine-7	Biolegend	1/200
CD69	Armenian Hamster IgG	H1.2F3	Pe	ImmunoKontact	1/100
CD69	Armenian Hamster IgG	H1.2F3	FITC	Biolegend	1/50
CD39	Rat IgG2b, κ	24DMS1	SB-436	BD Biosciences	1/200
CD103	Rat LOU IgG2a, κ	M290	APC-R700	BD Biosciences	1/200
Live/Dead Red	/	/	/	Thermofisher	1/200
Live/Dead ZG	/	/	/	Thermofisher	1/200

Table 1: **Antibodies used for T cell staining.** *These antibodies were used in 3 different panels in T cell experiments.*

Antibody anti-	Host / Isotype	Clone	Fluorochrome	Supplier	Dilution
CD8	Rat IgG2a, κ	53-6.7	APC	Biolegend	1/500
TCR	Rat IgG2a, λ	B20.1	Pe	BD Biosciences	1/100
TCR	Rat LOU IgG2a, λ	B20.1	APC-Cyanine-7	BD Biosciences	1/200
CD4	Rat IgG2b, κ	RM4-4	FITC	Biolegend	1/400
CD19	Rat IgG2a	6D5	Pe-Texas Red	Invitrogen	1/50
Dextramer	/	/	Pe	MBL International	1/5
Live/Dead violet	/	/	/	Thermofisher	1/200

Table 2: **Antibodies used for OT-I phenotyping.** *These antibodies were used in 2 different panels in T cell experiments.*

Antibody anti-	Host / Isotype	Clone	Fluorochrome	Supplier	Dilution
CD206	Rat IgG2a, κ	C068C2	FITC	Biolegend	1/50
CD68	Rat IgG2a	FA-11	Blue Violet 605	Biolegend	1/100
CD80	Armenian Hamster IgG	16-10A1	APC	Biolegend	1/100
CD86	Rat IgG2a, κ	GL-1	APCF750	Biolegend	1/100
Live/Dead violet	/	/	/	Thermofisher	1/200

Table 3: **Antibodies used for macrophage staining.** *These antibodies were used as a unique panel in macrophage experiments.*

2.9.1 Flow cytometry compensations (beads)

Flow cytometry results were compensated with VersaComp Antibody Capture Bead Kit (Beckman Coulter).

2.10 Quantification of inflammatory cytokines secretion

The quantification of cell expression was measured with ELISA kits. IFN-y secretion was measured on T cells with *Mouse IFN-y (AN-18) ELISA set* (BD OptEIA™) For VEGF secretion we used *ELISA mouse VEGF* (R&D Systems Inc). Finally TNF-a secretion was measured on polarized macrophages with *Mouse TNF (Mono/Mono) ELISA Set* (BD OptEIA™). Media used was separated from the 3 days post-treatment cells through centrifugation of V-plate at 1400 rcf for 5 minutes. For detailed ELISA biological procedures and principle, refer to section 3.2.

2.11 Determination of cAMP concentration

cAMP concentration was measured on T cells, M0 macrophages, Jurkat cells and Raw cells 3 days post-treatment using a Promega kit (Promega, cAMP-Glo™ Assay). For detailed biological principle refer to section 3.3.

2.12 Measure of gene expression

Gene expression of polarized macrophages under normoxia versus hypoxia was measured after seven days of generation plus three days of treatment. RNA was extracted using kit (A&A Biotechnology, Total RNA mini) and was turned into cDNA using TAKARA reverse transcription kit (TAKARA, PrimeScript™ RT Reagent Kit). Finally cDNA was analyzed by qPCR directly on CMU Genomic Core Platform. For detailed qPCR biological principle refer to section 3.4.

RNA of GBM human samples was extracted and processed using the same kits right after they have been thawed. Find below the detailed table of primers used for our experiments.

Primers	*Il-1b*	*Stat1*	*Nos2*	*Arg1*	*Mrc1*	*E-Cad*
Forward (F)	ACCCTGCA GCTG-GAGAGTGT	GCCTGGAT CAGCTGCA AAG	CCGG-CAAACCCA AGGTCT	GCAGAGG TCCAGAAG AATGG	TGTCAACC CTGCAGAT TTCAAG	GAGCGTG CCCCAG-TATCGT
Reverse (R)	CCATCTTCT TCTTTGGG TATTGCTT	GCTGCAG GGTCTCTG CAAC	CCGTGGA GCACGCTG AGTA	AGCATCCA CCCAAATG ACAC	GAGTGGCT TACGTGGT TGTTTC	GGCTGCCT TCAGGTTT TCATC

Table 4: **List of primers used for qPCR.** *Note that all the primers were mixed in two forms: one forward-specific strand (F) and one reverse-specific strand (R) transcription. As stated on introduction, Il-1b, Stat1 and Nos2 are mostly expressed on M1 macrophages while Arg1, Mrc1 and E-Cad are mainly expressed on M2 macrophages.*

2.13 Softwares

Kaluza Analysis Software (Beckman Coulter) was used for every flow cytometry analysis. Graphpad Prism was used to create the graphics and make the statistical analysis where Student tests were used for individual tests and ANOVAs were used for grouped tests. Finally, Biorender was used to create every original figure beside graphs.

3 Biochemical principles of the methods used

3.1 Flow cytometry analysis

Flow cytometry is a technique widely used in cellular biology, especially in oncology, haematology and stem cell research. The technique consists of lasers passing through a sample that has been pumped inside the cytometer and it gives information about the composition of the sample in order to discriminate the different cell populations (***Fig.7***).

Fluorescent antibodies are used to stain the cell membranes (membrane staining) or intracellular content (intracellular staining) before the analysis. Their detection gives information about the expression of certain markers and therefore the composition of the sample and its cell types.

Antibodies are chosen and used based on criteria of interest in a cell population. They are linked to fluorochromes that will emit fluorescence on a certain wavelength after they have been excited by the cytometer lasers. Antibodies and their associated fluorochromes have to overlay as less as possible to avoid a background on the results, that is why it is important to prepare a panel beforehand.

Data is then collected via an interphase tool-machine and is ready to analyse on the computer via a flow cytometry software. Kaluza software (Beckman Coulter) has been used for this analysis.

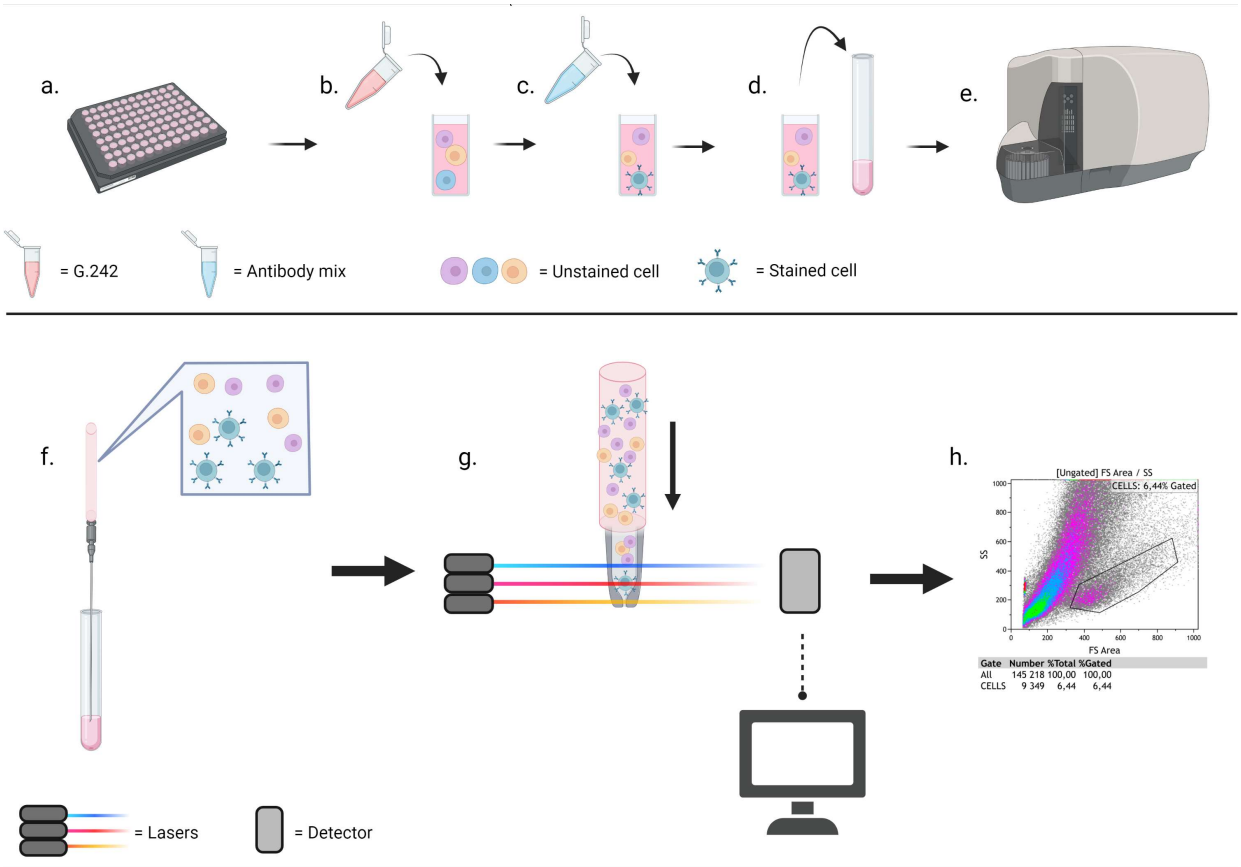


Figure 7: Flow cytometry preparation and principle. Set up; a. *Cells previously cultured and treated are plated on a V-plate;* ***b.*** *G.242 (FC.Block) is added in order to avoid unspecific interactions;* ***c.*** *Antibody mix (or isotype mix) is added. The antibodies are binding to their surface antigen;* ***d.*** *After a couple of washes, cells are transferred to analysis tubes;* ***e.*** *Tubes are placed on the carousel of the cytometer, ready to analyse.* ***Analysis; f.*** *Cells are pumped out of the tube by the cytometer;* ***g.*** *While they fall down a tube, lasers are passing trough them. The refraction of lasers and light emissions from excited fluorochromes is caught by a detector that computerize the information;* ***f.*** *Data is processed and analysed with a software.*

3.2 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a colorimetric method of analysis used to detect cell secretion within its medium. Different ELISA methods can be used with different applications and costs. The mains are: direct ELISA, indirect ELISA, Competitive ELISA and Sandwich ELISA.

In our case, the kits we used were sandwich ELISAs. The principle of this method is based on antibodies and light emission (***Fig.8***).

Captures antibodies specific to the protein of interest will be coated on a plate. After all cells have been removed, media is put inside the wells of the plate. If the protein is present it be captured by the antibodies. Another protein-specific is then added but this on is labelled with an enzyme that will react when we add the reagent. The reaction will make the substrate turn yellow if the protein of interest is present in a gradient-dependant way (more yellow if more proteins). After some time, a stop solution is added.

Finally, we read the plate with a microplate reader. It gives us the emission of light of the liquid. Data is then processed to find the concentration of every well. Concentration of samples is extrapolated via a standard curve.

This ELISA method is using two epitopes on the same target protein, making it more specific and accurate, compared to a direct, indirect or competitive ELISA that both use antibodies to one epitope.

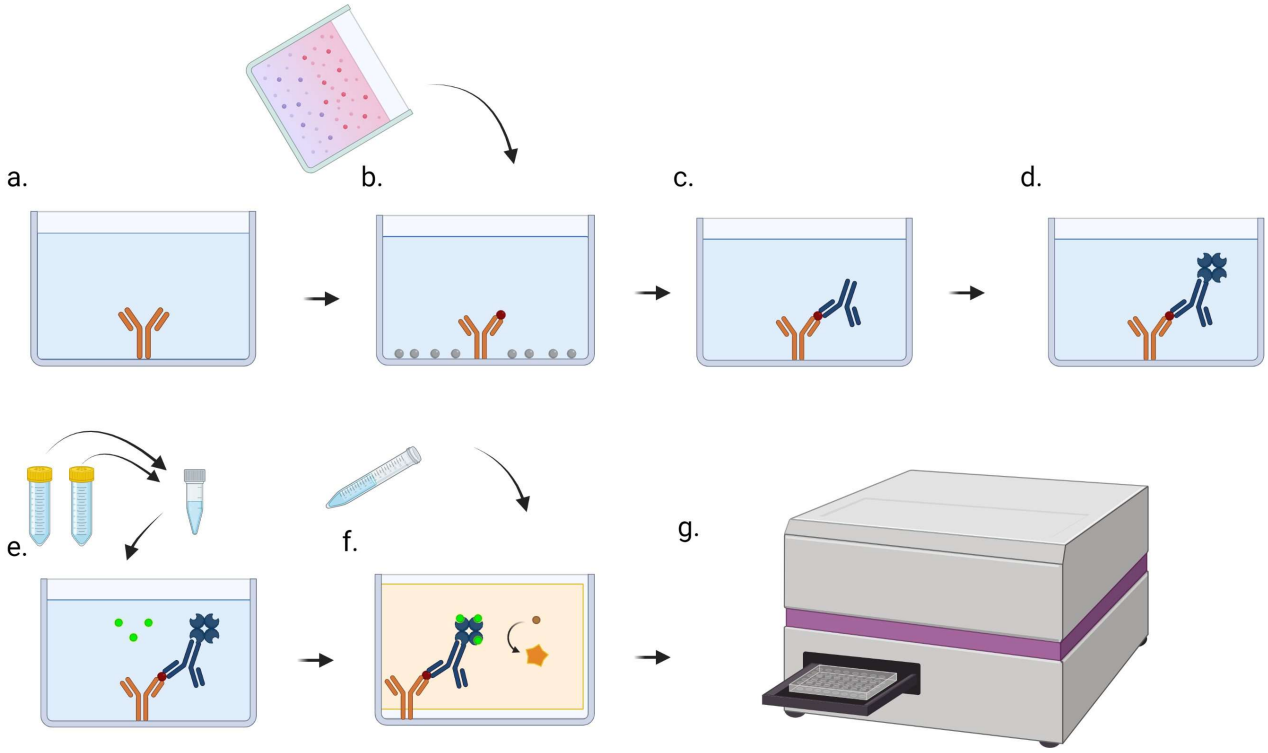


Figure 8: ELISA principle and main steps. a. *Coating with protein-specific antibody ;* ***b.*** *Media is added, protein is captured ;* ***c****. Detection antibody protein-specific is added and bind on a different epitope of the protein ;* ***d****. Enzyme reagent (streptavidin -horseradish) is added and bind to detection antibody ;* ***e****. Substrate reagent is added;* ***f.*** *Substrate binds to enzyme (peroxidase), the product of degradation turns the solution yellow, stop solution is then added;* ***g.*** *Plate is read via a microplate reader. Wells are washed a couple of time between every step.*

3.3 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) is a powerful method used in different fields of biology including virology, immunology and biotechnology. This is a great evolution of the classical PCR method.

A regular PCR is used to search for the presence of certain genes of interest within a sample after a lot of amplification cycles where DNA is duplicated thanks to a DNA polymerase. This method is therefore very sensitive and allows the user to detect known genes at very low concentration, in the case of a viral infection for example. This is a qualitative method that gives information only about presence or absence of a fragment of DNA of interest within a sample.

qPCR, in contrast to PCR, is a quantitative analysis that detects, characterizes and quantifies a given gene of interest within a sample. In qPCR fluorophores-probe constructs are used to mark the newly synthesised DNA after each cycle of replication, that way it is possible to have a real-time quantification of the sample. Probe with fluorophore will bind to the double strand DNA, then when the polymerization reaches the probe it emits fluorescence. The more probes bind the more fluorescent it get, giving information about the concentration (***Fig. 9***).

Furthermore, housekeeping genes are used in qPCR. They are genes coding for ubiquitous cell functions. They can therefore be used in order to normalize the qPCR analysis. They are essential in order to improve the quality of the cDNA and decrease the errors and contaminations from the RNA extraction steps.[73]

Both are based on the same principle and basis. DNA will be separated in two strands (denaturation via heat) and duplicated via DNA polymerases that will fix to primers (short DNA sequences needed to start the replication) and elongates the strands, then the cycle starts over again. The quantity of DNA of interest is increasing exponentially. Note that it is possible to use RNA both in PCR and qPCR however a reverse transcription is needed in order to get the corresponding complementary DNA (cDNA).

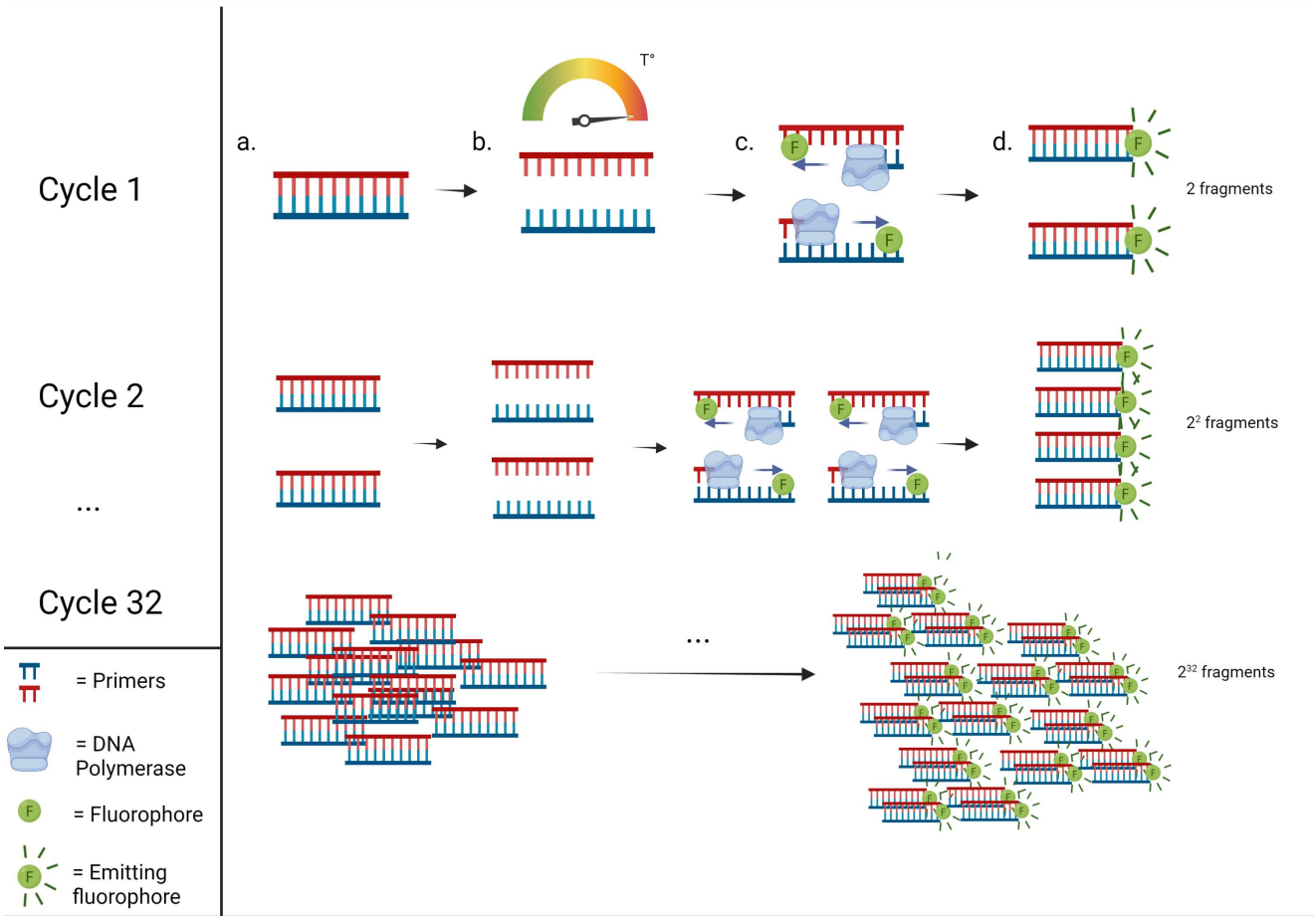


Figure 9: qPCR principle and main steps. a. *Double strand DNA is isolated or obtained through reverse transcription of RNA ;* ***b.*** *DNA is heated in order to denature it and obtain 2 single strands DNA ;* ***c.*** *primers (forward and reverse) are fixed to their specific complementary region. DNA polymerases anchors to these primers and starts replication ;* ***d.*** *The fragments are completely replicated. The fluorophore molecules previously fixed to the end of the fragments have been activated by the polymerase during replication. There is light emission, first cycle is completed, cycle 2 starts.* ***[a-d]*** *Represents a complete cycle. Light emission is monitored after each cycle.*

3.4 Cyclic Adenosine Monophosphate assay

Cyclic Adenosine Monophosphate (cAMP) is a second messenger important in many cell processes, including signal transduction. This is a derivative of ATP, therefore an efficient and more direct way to explore and measure the ADO effect on cells. The method is based on the cAMP cascade (***Fig.10***). Remaining ATP is measured through a Luciferin-Luciferase complex that emits bioluminescence while activated in presence of ATP.[74]

Note that the measured luminescence is inversely proportional to the level of cAMP, therefore a strong luminescence means a low level of cAMP and a low luminescence means a high level of cAMP (ATP needed for the Luciferin-Luciferase complex to produce bioluminescence has been turned to cAMP by the adenylate cyclase).

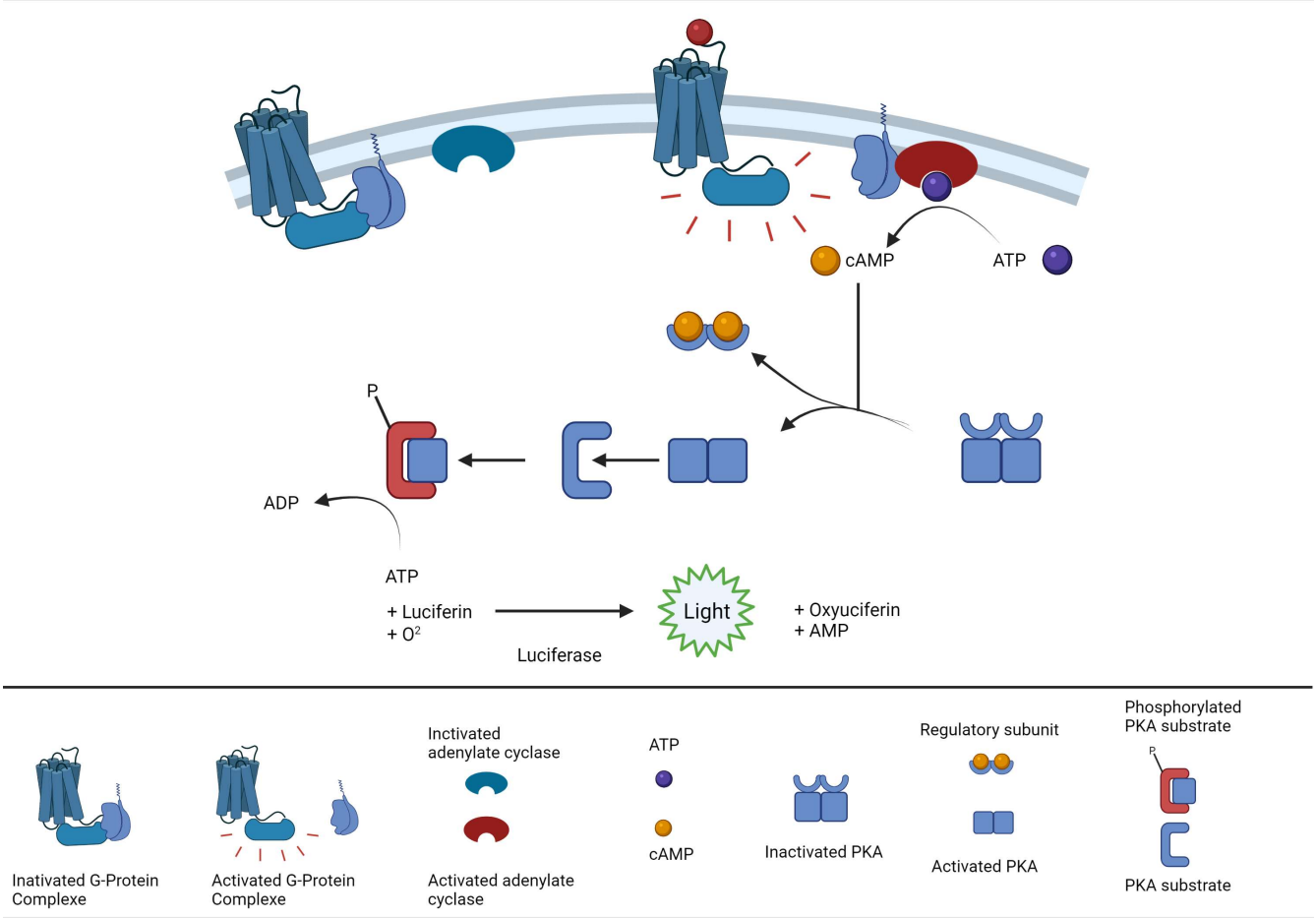


Figure 10: cAMP assay principle. *Extracellular ATP binds to its specific G-protein complex. The G-protein α subunit moves and binds to adenylate cyclase and activates it. Intracellular ATP is turned to cAMP by the activated adenylate cyclase and binds to the regulatory subunits (dimers) of the protein kinase A (PKA), the regulatory subunits gone, PKA can now phosphorylate its substrate, consuming ATP in the process. With the kit, the ATP that is not consumed in the process will react with luciferin, O_2 and be turned to light emissions, oxyluciferin and AMP by the luciferase.*

4 Results

4.1 Heterogeneity shown through cancer cell gene and protein expression

As mentioned earlier, the intra/inter tumour heterogeneity is a key aspect of the partial inefficiency of GBM treatments. As we stated on section ***1.8***, CD39 and CD73 are surface proteins whose role is to turn ATP to ADO and that are upregulated in the context of cancer because of the hypoxic microenvironment. A2a receptor will therefore be upregulated as well in response to the increase of ADO and same goes with SLC2 (glucose transporter) due to the high demand of glucose from cancer cells because of their incapacity to do oxidative phosphorylation and the fact they rely on glycolysis only for their energy supply.[44] Carbonic anhydrase IX (CA9) is protein whose role is to maintain the intracellular pH, altered by hypoxic microenvironment, its gene will therefore be hypoxia-induced and upregulated in context of cancer.[75]

All these genes can therefore be used as markers of hypoxia and glucose consumption to characterize tumours. We will see that the expression pattern can vary a lot between the patients, highlighting GBM heterogeneity. (***Fig.11A***).

We also mentioned that tumours secrete VEGF in order to increase the blood supply needed to bring the nutrients (glucose) that cancer cells need to run. Due to the fact cancer cells rely on glycolysis-only we would expect their VEGF secretion to be upregulated under hypoxic environment. We tested this and saw that different cancer cell lines do not respond equally to low oxygen environment (***Fig.11B***).

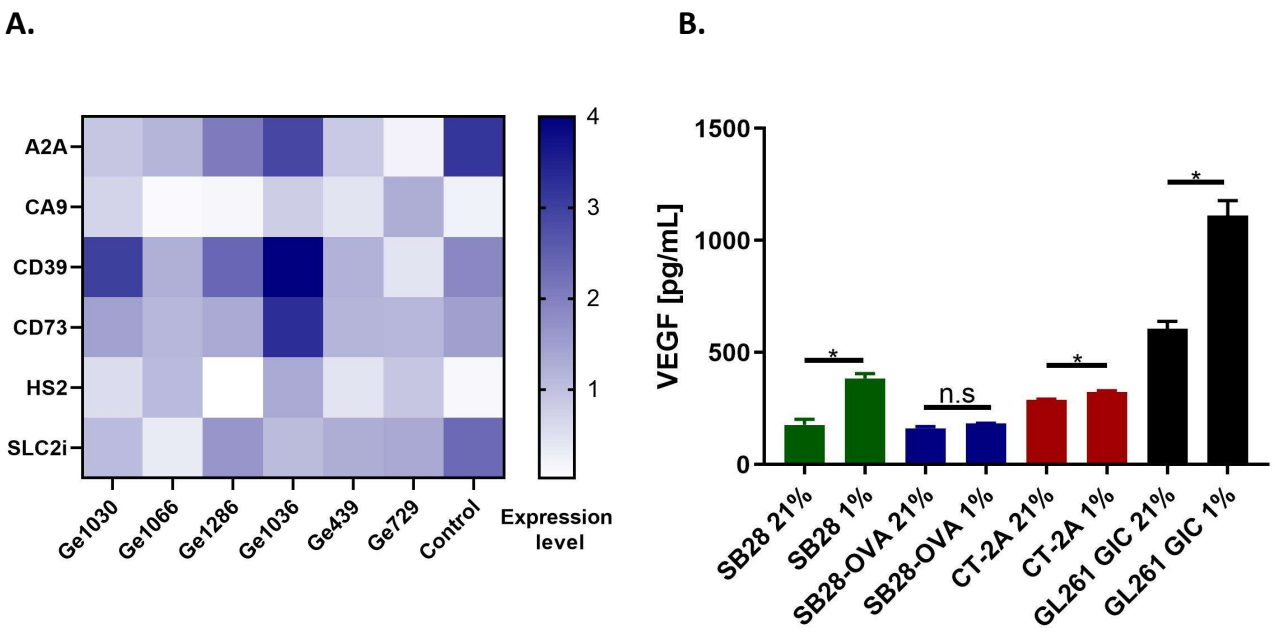


Figure 11: Differential gene and protein expression of glioma samples and cancer cell lines under hypoxia. A. *Six samples from different patients with high-grades gliomas were analysed through qPCR for six genes commonly monitored in cancer samples (A2A, CA9, CD39, CD73, H2S, SLC2i). We used a sample from an epileptic patient as a control.* ***B.*** *Four different cancer cell lines (SB28, SB28-OVA, CT-2A, GL261) were cultured in 21% and 1% O_2, their VEGF secretion was measured with an ELISA kit after one week of culture; * p≤* 0.05.

In both experiments we can observe the tumour heterogeneity. None of the patients showed a similar pattern of secretion even though certain are closer than other (Ge1286-Ge1036)(***Fig.11A***). There were differences of secretion between the two conditions in all the cell lines (only SB28-OVA was not significant), with a predictable overexpression in the 1% O_2 condition but different order of magnitude (+118% in SB28, +12% in SB28-OVA, +12% in CT-2A, +85% in GL261 GIC)(***Fig.11B***).

4.2 Adenosine and hypoxia effects on Macrophage polarization

4.2.1 Effects of ADO and hypoxia on the expression of different surface markers

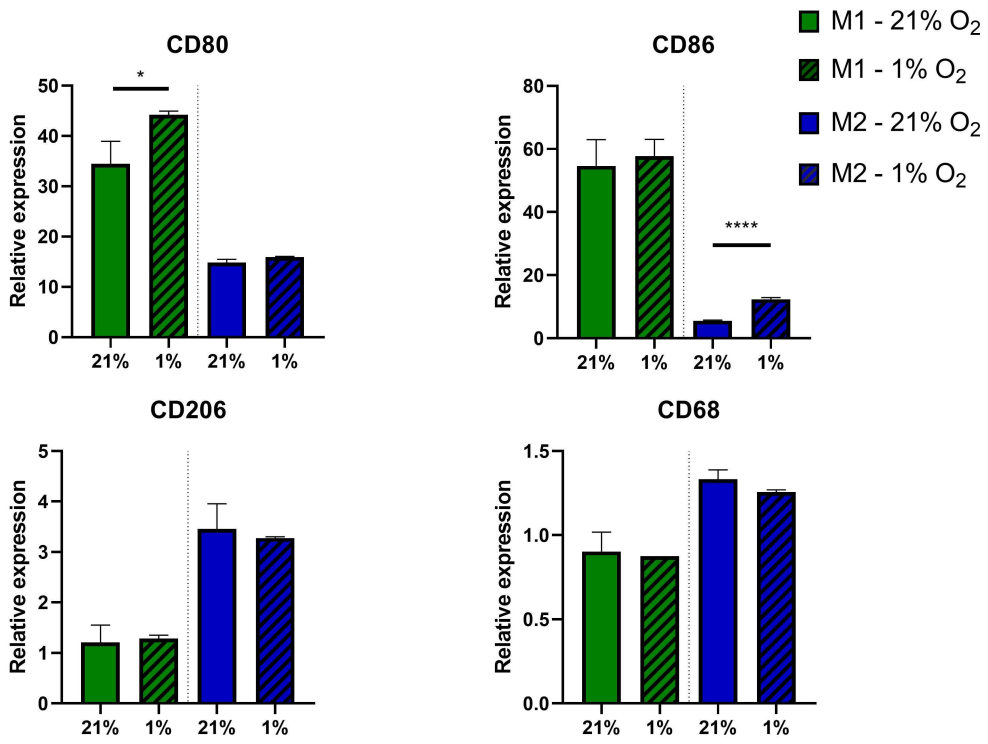


Figure 12: Hypoxia-only induces expression of certain M1 markers. *Polarized M1 and M2 macrophages were cultured under normoxia and hypoxia for three days. The present results are issued from flow cytometry analysis, using immunofluorescent surface antibodies (anti-CD80, anti-CD86, anti-CD206, anti-CD68) where untreated cells were gated on singulet live macrophages. The results are the relative expression divided isotype. Statistical analysis was done using GraphPad Prism paired parametric T test; * p≤* 0.05 ; **** p≤0.0001.

CD80 and CD86 were used as M1 markers while CD206 and CD68 were used as M2 markers for the present and next experiments. This figure serves as a quality control to confirm that our macrophages were still polarized after three days and observe how hypoxia-only affect the expression of these surface markers. The present results (***Fig.12***) confirmed that our M1 macrophages, as expected, highly express CD80/CD86 while M2 macrophages do not. On the other hand, M2 macrophages express three times more CD206 compared to M1 macrophages and expressed more CD68 as well. Interestingly we see that hypoxia-only, without addition of any compound, makes M1 macrophages significantly overexpress CD80 (+28%) and makes M1 macrophages significantly overexpress CD80 (+120%), slightly pushing them more toward a M1-like phenotype.

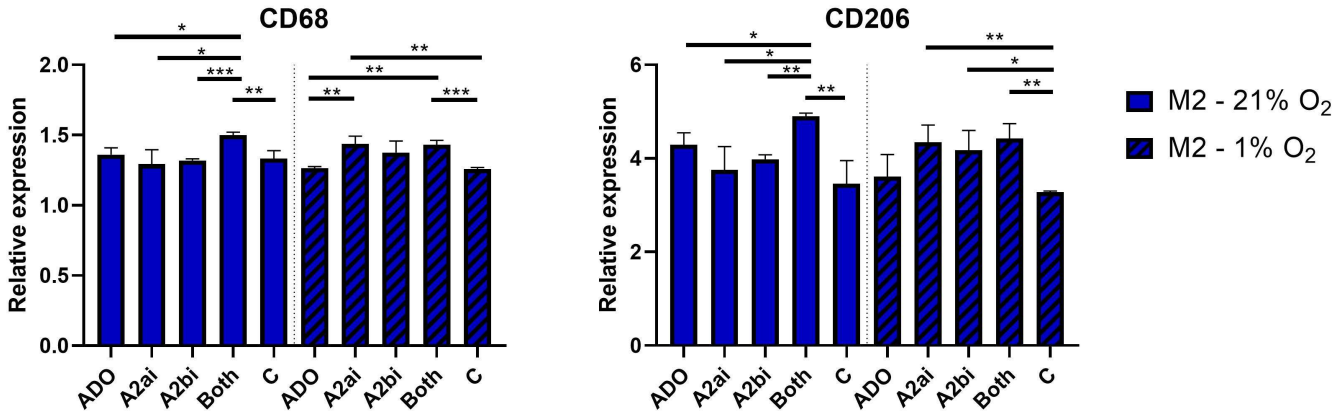


Figure 13: ADO inhibitors but not ADO itself upregulate M2 marker expression on M2 macrophages. *ADO: Adenosine; A2ai: ADO + A2ai; A2bi: ADO + A2bi; Both: ADO + A2ai + A2bi; C: Control (Untreated cells). Polarized M1 and M2 macrophages were cultured with Adenosine and Adenosine inhibitors under normoxia and hypoxia for three days. The present results are issued from flow cytometry analysis, using immunofluorescent surface antibodies (anti-CD206, anti-CD68) where cells were gated on singulet live macrophages. The results are the relative expression divided isotype. Statistical analysis was done using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p*≤0.01; *** p≤0.001.*

Based on reports from literature, we expected ADO to make all macrophages overexpress M1 markers such as CD68 and CD206, however it was not observed in M1 nor M2 macrophages (***Fig.13***). M1 macrophages presented no significant differences in any of the conditions and the expression of both markers was very low so we decided to not include the data here. M2 macrophages however shown a similar unexpected pattern of expression for both CD68 and CD206. The addition of ADO did not increase the expression of CD68 or CD206, however both markers were significantly overexpressed in normoxia and hypoxia in presence of ADO inhibitors. The combination of both inhibitors (A2ai and A2bi) presented the highest increase for both markers compared to control with +30% (normoxia) and +27% (hypoxia) overexpression of CD206 and +12% (normoxia) and +13% (hypoxia) overexpression of CD68.

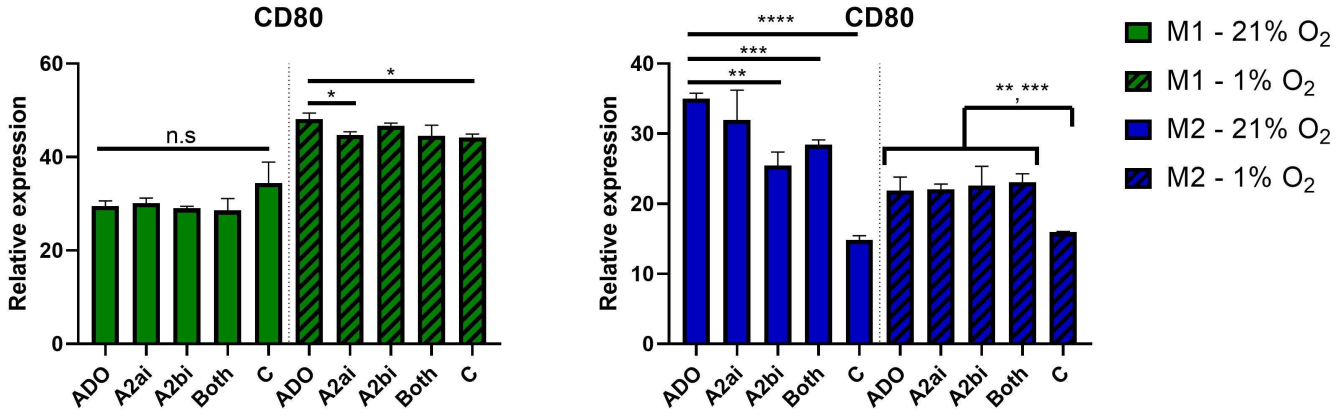


Figure 14: Hypoxia and ADO but not inhibitors induce CD80 overexpression. *ADO: Adenosine; A2ai: ADO + A2ai; A2bi: ADO + A2bi; Both: ADO + A2ai + A2bi; C: Control (Untreated cells). Polarized M1 and M2 macrophages were cultured with Adenosine and Adenosine inhibitors under normoxia and hypoxia for three days. The present results are issued from flow cytometry analysis, using immunofluorescent surface antibodies (anti-CD80) where cells were gated on singulet live macrophages. The results are the relative expression divided isotype. Statistical analysis was done using GraphPad Prism paired parametric T test for individual comparisons and two-way ANOVA for groups comparisons; * p≤* 0.05 ; ** p*≤0.01; *** p≤0.001;* **** p≤0.0001.

As we expected ADO to downregulate M1 markers expression, we also expected hypoxia to produce a similar effect. None of these effects was observed (***Fig.14***). In M1 macrophages we observed no difference within normoxic conditions, however a significant general overexpression of CD80 was detected in hypoxia for all groups compared to normoxia ($p<0.0001$). Adenosine also upregulated this expression in hypoxia, while only A2ai slightly reduced it. A different tendency was observed among M2 macrophages. As mentioned earlier, we observed no difference between control groups from normoxia and hypoxia on M2 macrophages, however we report a significant general decrease in hypoxia versus normoxia for all the other conditions ($p<0.05$). Furthermore, ADO increased CD80 expression on normoxic M2 macrophages by +136%, this overexpression was reduced by both inhibitors. A2bi had the biggest effect and reduced the overexpression from +136% to +69%. Finally, in hypoxia ADO significantly increased CD80 expression by +37% but none of the inhibitor conditions managed to counterbalance this effect, all the conditions were higher than control while not being different than ADO.

To conclude, hypoxia seems to have different effects on M1 and M2 macrophages. It upregulates CD80 on M1 macrophages while ADO and the inhibitors did not affect the expression much. The pattern on M2 macrophages was opposite as ADO seems to be a necessary compound for CD80 overexpression while hypoxia, in this case, lowered its expression.

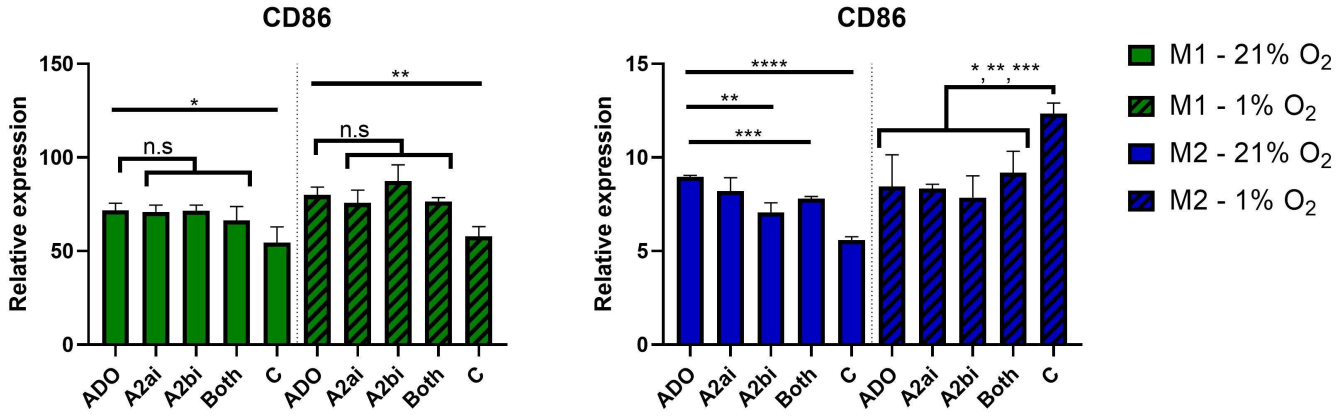


Figure 15: Adenosine increase CD86 expression in normoxia but not in hypoxia. *ADO: Adenosine; A2ai: ADO + A2ai; A2bi: ADO + A2bi; Both: ADO + A2ai + A2bi; C: Control (Untreated cells). Polarized M1 and M2 macrophages were cultured with Adenosine and Adenosine inhibitors under normoxia and hypoxia for three days. The present results are issued from flow cytometry analysis, using immunofluorescent surface antibodies (anti-86) where cells were gated on singulet live macrophages. The results are the relative expression divided isotype. Statistical analysis was done using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p*≤0.01; *** p≤0.001;* **** p≤0.0001.

Similarly with our previous observations on CD80, we observed that ADO significantly increased CD86 expression in M1 macrophages both in normoxia and hypoxia, respectively by +31% and +38% (***Fig.15***). This effect was not reduced by any of our inhibitors. In M2 normoxic macrophages, a similar overexpression was observed (+60% overexpression), however this overexpression was partially counterbalanced by the inhibitors. A2bi presented the biggest change as it significantly reduced the overexpression from +60% to +39% compared to control. Under hypoxia we observed an opposite tendency: ADO significantly reduced CD86 expression by -32% while none of the inhibitor conditions were different from ADO.

Overall, we again observed a mostly upregulative effect from ADO addition, while the inhibitors effect was limited to M2 normoxic macrophages. Hypoxia seems to slightly upregulate CD86 expression on all macrophages subtypes with a bigger impact on hypoxic M2 macrophages.

4.2.2 Effects of ADO on intracellular cAMP concentration on Raw macrophages

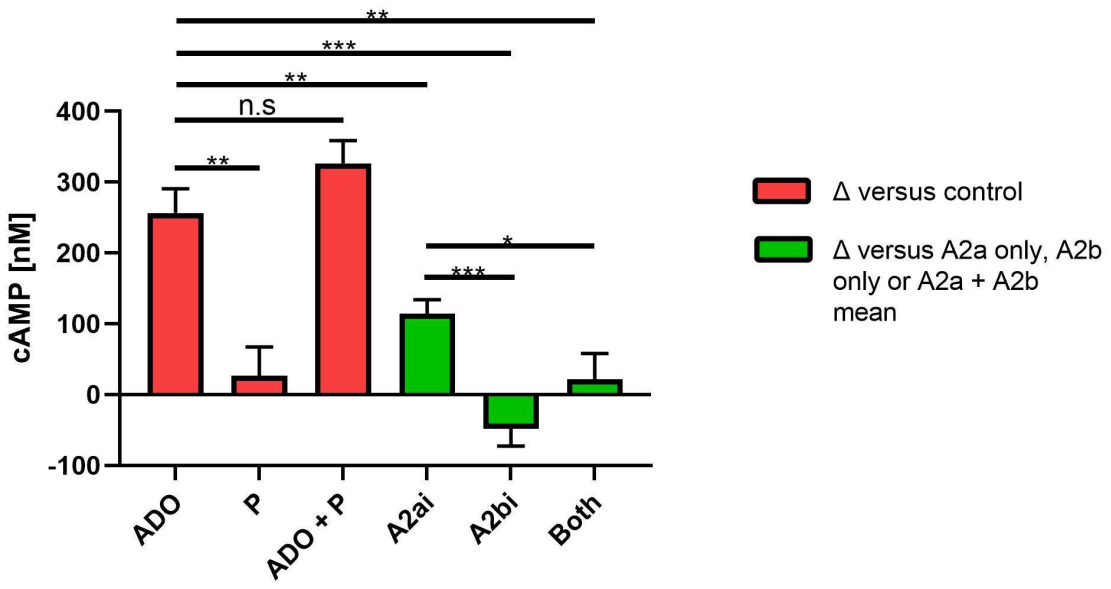


Figure 16: A2ai and A2bi efficiently reduce cAMP generated by extracellular ADO addition in Raw cells. *ADO: Adenosine; P: Pentostatin; ADO + P: Adenosine + Pentostatin; A2ai: Adenosine + A2ai; A2bi: Adenosine + A2bi; Both: Adenosine + A2ai + A2bi. Raw cells were thawed, cultured for one week then passed. Cells were treated after a first passage and incubated at 21% O_2 for three days whereafter their media was taken for this analysis. Results are issued from cAMP assay and represent the delta versus untreated cells (red) or versus vehicle control (inhibitors, green). Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p*≤0.01; *** p≤0.001.*

cAMP generation was monitored on Raw cells in order to have a more direct quantitative overview of the effect of our inhibitors on ADO on a macrophage cancer cell line since extracellular ADO induce cAMP generation. Pentostatin is an ADA inhibitor, lowering the ADO to Inosine conversion (refer to ***Fig.2***). We therefore expected Pentostatin to exacerbate ADO stability and effects when mixed together. As we predicted, a higher cAMP level on A+P group was observed in comparison to ADO-only (***Fig.16***), while the Pento-only remained very low. On the other hand, both our inhibitor and the combo showed a significant reduction of cAMP level compared to ADO, A2bi being noticeably more effective than A2ai, as we could expect from the literature. It presented an even lower concentration than the control group (negative delta) which makes sense as ADO (and cAMP) are constitutively present in all cells.

These results bring us two important findings: first, it confirms that ADA plays a substantial role in the ADO (and cAMP) equilibrium, therefore confirms its interest as a target. We also validated that the inhibition of A2r, especially A2br is a potent way to reduce the cAMP, therefore ADO concentration.

4.2.4 Adenosine and hypoxia modify the gene signature of polarized macrophages

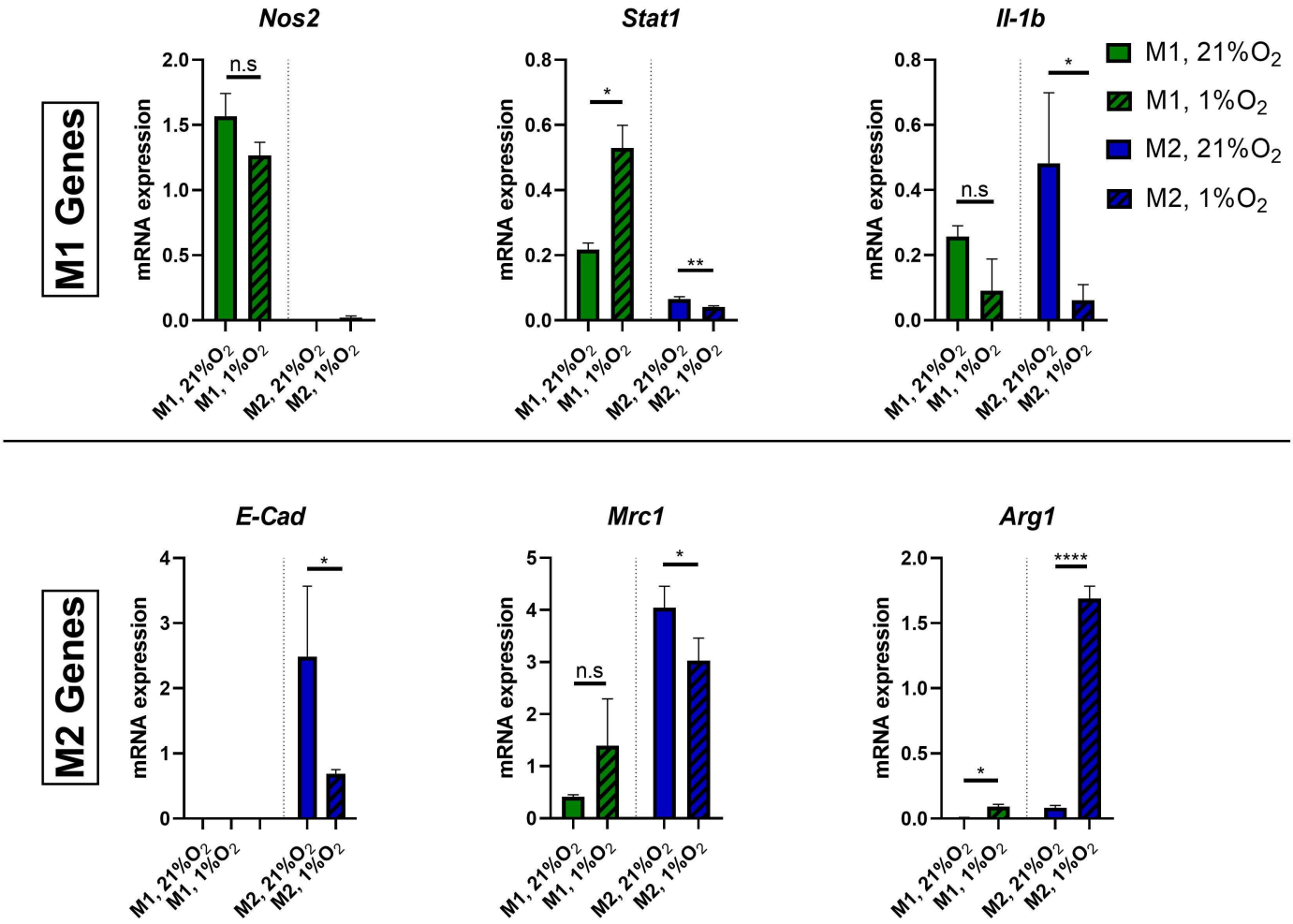


Figure 17: Hypoxia exerts heterogeneous effects on macrophage gene expression. *The present data are the results of qPCR analysis on polarized macrophages. After polarization M1 and M2 macrophages were incubated in normoxia and hypoxia for three days. RNA was extracted using Total RNA mini kits (A&A Biotechnology) and turned to cDNA using TAKARA protocol. Results represent the mRNA expression of 6 polarization signature genes (Nos2, Stat1, Il-1b, E-Cad, Mrc1, Arg1) compared to 2 housekeeping genes (Eef1a1, Gapdh) after 32 cycles of qPCR. Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p≤*0.01;* **** p≤0.0001.

Expression from the control condition (untreated cells) at both oxygen saturation was measured in order to check the polarized state of our macrophages under normoxia, three days post generation. It also permits to measure the effect of hypoxia-only on macrophage polarization (***Fig.17***). First of all, under hypoxia we see that, excluding *Il-1b*, all our M1 markers were expressed by M1 macrophages but not (or at very low levels) by our M2 macrophages. On the other hand, all our M2 markers were expressed by M2 macrophages

but not (or at very low levels) by M1 macrophages. This confirms the intended polarization of our macrophages as a quality control for the other conditions.

It is interesting to note that hypoxia, in the absence of any compound was already shifting most of the gene expression but there is not a clear trend. Indeed, while all M1-like genes were downregulated with hypoxia, *Stat1* was significantly upregulated only in hypoxic M1 macrophages. Interestingly, *E-Cad* and *Mrc1* stayed unexpressed in hypoxic M1 macrophages but were significantly downregulated in hypoxic M2 macrophages while *Arg1* was upregulated both in M1 (from no expression to low expression) and M2 (+2000%) macrophages.

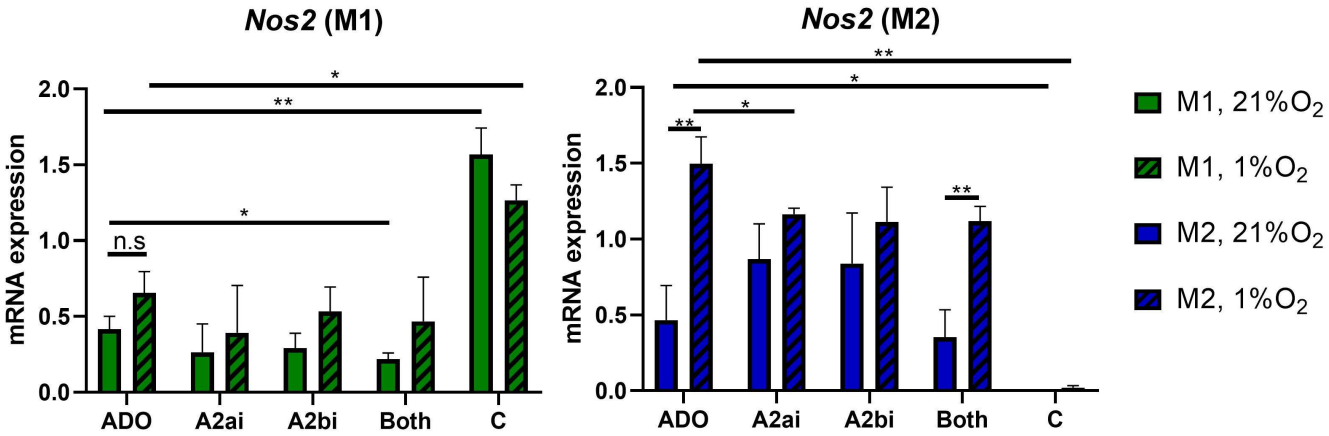


Figure 18: Hypoxia combined with ADO makes M2 macrophages express similar levels of Nos2 compared to untreated M1 macrophages. *ADO: Adenosine; A2ai: ADO + A2ai; A2bi: ADO + A2bi; Both: ADO + A2ai + A2bi; C: Control (Untreated cells). The present data are the results of qPCR analysis on polarized macrophages. After polarization M1 and M2 macrophages were treated and incubated in normoxia and hypoxia for three days. RNA was extracted using Total RNA mini kits (A&A Biotechnology) and turned to cDNA using TAKARA protocol. Results represent the mRNA expression of Nos2, a M1 marker, compared to 2 housekeeping genes (Eef1a1, Gapdh) after 32 cycles of qPCR. Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p*≤0.01.*

We observed different reactions to ADO from our macrophages in regards to *Nos2* expression (***Fig.18***). On one hand, M1 macrophage-controls had their *Nos2* expression significantly reduced by the addition of ADO both in normoxic and hypoxic conditions. On the other hand the hypoxia itself induces a nonsignificant increase of expression in every condition except control. Finally, our inhibitor reduced *Nos2* expression in all the conditions compared to ADO.

M2 macrophages presented an opposite but yet very interesting effect. M2 macrophages when untreated expressed none to very low amount of *Nos2*. However, when ADO was

added, the expression drastically increased. This effect was even more important when cells were cultured in hypoxia in the presence of ADO to the extend that M2 macrophages hypoxic-ADO were expressing as much *Nos2* as M1 macrophages normoxic-Control. Hypoxia itself did not significantly changed *Nos2* expression, however there is a clear trend that suggests that Hypoxia is upregulating its expression. As instance, in presence of ADO, we observed three times fold more *Nos2* in M2 macrophages hypoxic-ADO compared to M2 macrophages normoxic-ADO. Finally, no significant difference was observed on M2 macrophages with the addition of inhibitor compared to ADO in normoxia, nonetheless, in hypoxia all the inhibitors reduced the overexpression induced by ADO with A2ai being the only one presenting a significant difference.

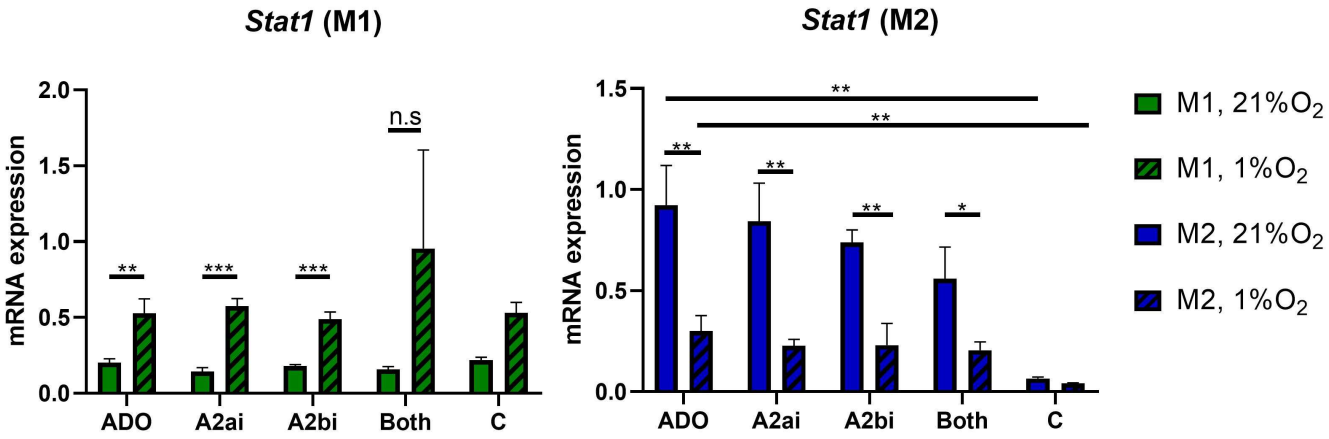


Figure 19: Hypoxia makes M1 but not M2 macrophages overexpress Stat1. *ADO: Adenosine; A2ai: ADO + A2ai; A2bi: ADO + A2bi; Both: ADO + A2ai + A2bi; C: Control (Untreated cells). The present data are the results of qPCR analysis on polarized macrophages. After polarization M1 and M2 macrophages were treated and incubated in normoxia and hypoxia for three days. RNA was extracted using Total RNA mini kits (A&A Biotechnology) and turned to cDNA using TAKARA protocol. Results represent the mRNA expression of Stat1, a M1 marker, compared to 2 housekeeping genes (Eef1a1, Gapdh) after 32 cycles of qPCR. Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p≤*0.01; *** p≤0.001;* **** p≤0.0001.

Similarly to *Nos2* results, *Stat1* results are very interesting yet heterogeneous between M1 and M2 macrophages. Indeed, hypoxia-only was sufficient to significantly upregulate *Stat1* expression in every condition for M1 macrophages (***Fig.19***). In M1 macrophages no difference was observed between ADO and Control neither in normoxia nor in hypoxia, same goes for the inhibitors.

In contrast to M1 macrophages that overexpressed *Stat1* in hypoxia, M2 macrophages were significantly overexpressing *Stat1* in normoxia compared to hypoxia in all the conditions. The addition of ADO significantly increased the expression compared to control

both in normoxia and hypoxia. Finally, our inhibitors nonsignificantly reduced this overexpression.

Hypoxia seems to be a major actor in the regulation of *Stat1* as it was the only factor that mattered in M1 macrophages expression (upregulation) and it was also coacting with ADO in M2 macrophages to regulate *Stat1* expression (downregulation).

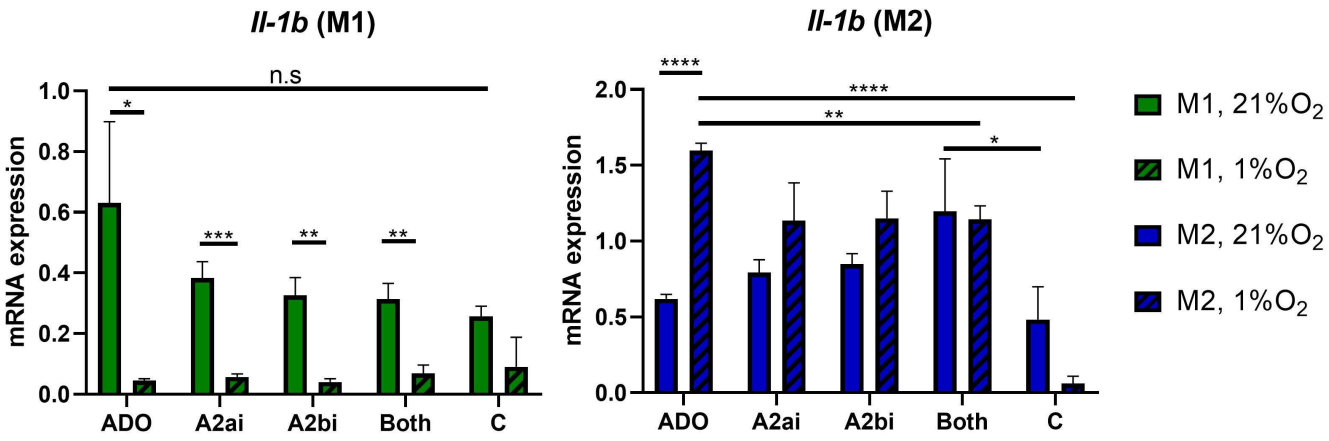


Figure 20: Hypoxia reduce Il-1b expression in M1 but not in M2 macrophages. *ADO: Adenosine; A2ai: ADO + A2ai; A2bi: ADO + A2bi; Both: ADO + A2ai + A2bi; C: Control (Untreated cells). The present data are the results of qPCR analysis on polarized macrophages. After polarization M1 and M2 macrophages were treated and incubated in normoxia and hypoxia for three days. RNA was extracted using Total RNA mini kits (A&A Biotechnology) and turned to cDNA using TAKARA protocol. Results represent the mRNA expression of Il-1b, a supposed M1 marker, compared to 2 housekeeping genes (Eef1a1, Gapdh) after 32 cycles of qPCR. Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p≤*0.01; *** p≤0.001;* **** p≤0.0001.

Il-1b is reported in literature as being part of M1 macrophage gene signature, however, as mentioned earlier and contrary to our M2 macrophages, our M1 macrophages, did not expressed high amounts of *Il-1b*. The already low *Il-1b* was even lower when M1 macrophages were cultured in hypoxia (***Fig.20***). This was confirmed and significant for all the conditions except control. Interestingly, while hypoxia reduces M1 macrophages *Il-1b* expression, it seems that ADO upregulates this expression and inhibitors tend to reduce it. Results not being significant, these observations need further experiments to be confirmed but the possibility that ADO might counteract some of hypoxia effects is interesting and needs to be explored.

Effects on M2 macrophages are very heterogeneous compared to M1 macrophages. First of all we notice that ADO and Control are not different in M2 macrophages normoxic conditions while inhibitors seem to upregulate *Il-1b* expression. When it comes to hypoxia,

the observations are however different. First of all, the combination of hypoxia + ADO makes M2 macrophages highly overexpress *Il-1b* (compared to ADO-normoxia and Control-hypoxia). Because expression was close to zero in hypoxic control plus ADO and Control were not significantly different in normoxic samples, this overexpression probably requires both hypoxia and ADO simultaneously to be triggered. Finally, we see that the inhibitors reduced the increase due to ADO + hypoxia but did not affect the normoxic samples.

Once again, hypoxia seems to be a major actor in *Il-1b* regulation as it had impact on both M1 and M2 macrophage populations with opposite effects and acted in concert with ADO on M2 macrophages.

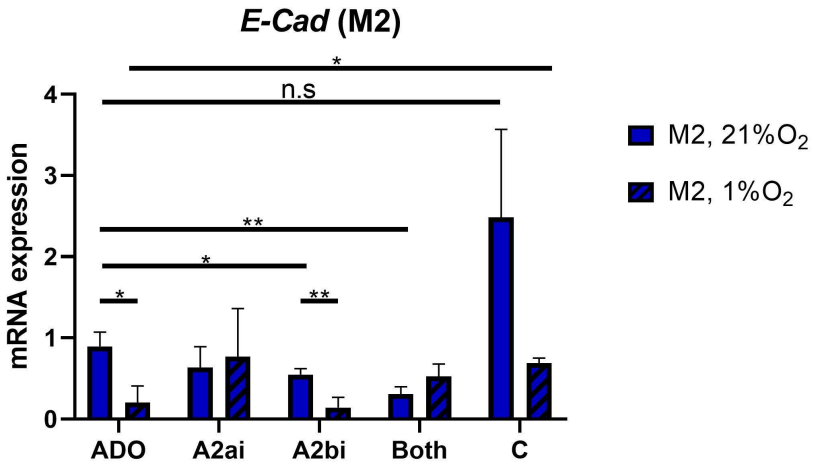


Figure 21: ADO and hypoxia are downregulating E-Cadherin expression on M2 macrophages. *ADO: Adenosine; A2ai: ADO + A2ai; A2bi: ADO + A2bi; Both: ADO + A2ai + A2bi; C: Control (Untreated cells). The present data are the results of qPCR analysis on polarized macrophages. After polarization M1 and M2 macrophages were treated and incubated in normoxia and hypoxia for three days. RNA was extracted using Total RNA mini kits (A&A Biotechnology) and turned to cDNA using TAKARA protocol. Results represent the mRNA expression of E-Cad, a M2 marker, compared to 2 housekeeping genes (Eef1a1, Gapdh) after 32 cycles of qPCR. Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p≤*0.01.*

We did not detect *E-Cad* expression in M1 macrophages, regardless of the oxygen tension and the treatment applied, confirming their polarization status.

Surprisingly, on M2 macrophages the hypoxia induced a reduced expression compared to normoxia, this effect was significant on ADO, A2bi and Control group (***Fig.21***). Additionally we observed that ADO itself was also reducing the expression when comparing ADO-normoxic with Control-normoxic and ADO-hypoxic with Control-hypoxic. Finally, we see that A2bi significantly lowered the expression compared to ADO, this effect was only observed in normoxia both in A2bi and “Both” groups. Contrary to the previous markers,

hypoxia and extracellular ADO seem to push *E-Cad* expression toward the same direction which is a global downregulation on cells that were physiologically expressing it (M2).

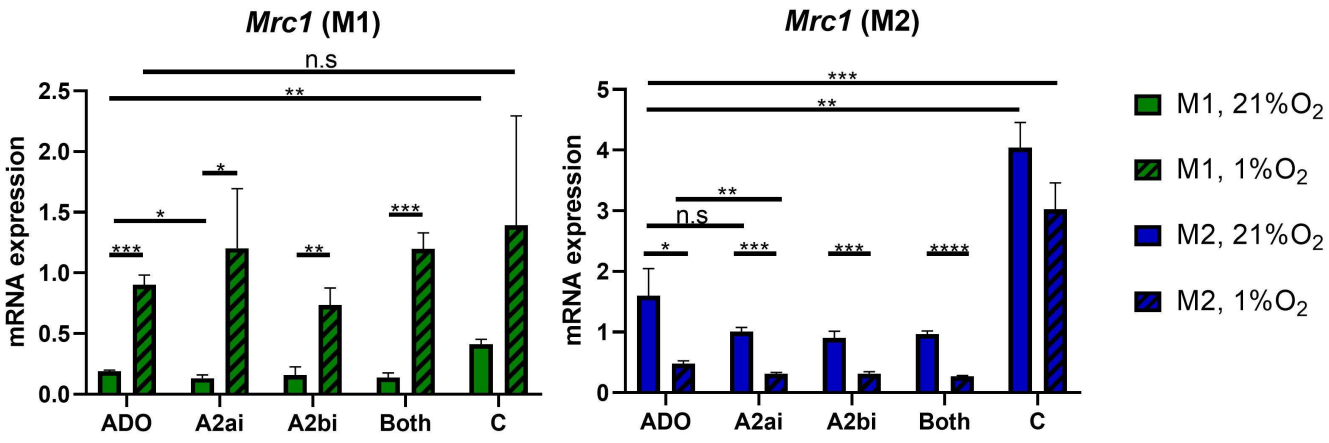


Figure 22: ADO downregulates Mrc1 expression in M1 and M2 macrophages but hypoxia has two opposite effects in these cells. *ADO: Adenosine; A2ai: ADO + A2ai; A2bi: ADO + A2bi; Both: ADO + A2ai + A2bi; C: Control (Untreated cells). The present data are the results of qPCR analysis on polarized macrophages. After polarization M1 and M2 macrophages were treated and incubated in normoxia and hypoxia for three days. RNA was extracted using Total RNA mini kits (A&A Biotechnology) and turned to cDNA using TAKARA protocol. Results represent the mRNA expression of Mrc1, a M2 marker, compared to 2 housekeeping genes (Eef1a1, Gapdh) after 32 cycles of qPCR. Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p≤*0.01; *** p≤0.001;* **** p≤0.0001.

Mrc1 expression in M1 macrophages was reduced in normoxia and hypoxia with the addition of ADO (***Fig.22***). Interestingly, this expression was significantly upregulated with hypoxia compared to normoxia in every condition. No noticeable differences was observed within hypoxic conditions, however a slight but significant reduction was seen in normoxic A2ai compared to ADO.

Contrary to M1 macrophages, where hypoxia upregulated *Mrc1* expression, in M2 macrophages, hypoxia significantly downregulated this expression in all the conditions. Similarly with M1 macrophages results, ADO lowered *Mrc1* expression in hypoxia and normoxia, however the inhibitors did not compensate the downregulation but worsen it even more.

Overall, all the normoxic M2 macrophages expressed more *Mrc1* than the normoxic M1 macrophages, however the hypoxia seems to again have a central role in the regulation of this gene, as it completely inverted the trends on our macrophages populations: under hypoxia M1 macrophages expressed more *Mrc1* than M2 macrophages.

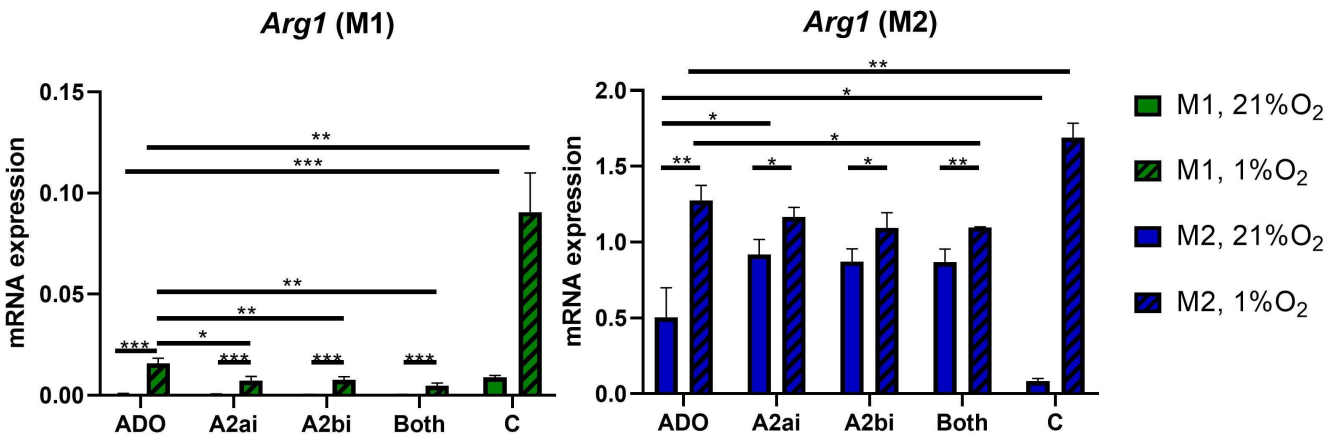


Figure 23: Hypoxia upregulates Arg1 expression on M1 and M2 macrophages. *ADO: Adenosine; A2ai: ADO + A2ai; A2bi: ADO + A2bi; Both: ADO + A2ai + A2bi; C: Control (Untreated cells). The present data are the results of qPCR analysis on polarized macrophages. After polarization M1 and M2 macrophages were treated and incubated in normoxia and hypoxia for three days. RNA was extracted using Total RNA mini kits (A&A Biotechnology) and turned to cDNA using TAKARA protocol. Results represent the mRNA expression of Arg1, a M2 marker, compared to 2 housekeeping genes (Eef1a1, Gapdh) after 32 cycles of qPCR. Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p≤*0.01; *** p≤0.001*.

To conclude this qPCR analysis, our interest was directed toward *Arg1* (***Fig.23***). First of all, on M1 macrophages, we see that hypoxia significantly increased *Arg1* expression in Control (from very low to low expression) as well as the other conditions (from none to very low expression). We also see that ADO downregulated this expression compared to Control both in hypoxia and normoxia, while inhibitors reduced even more than ADO alone (p≤0.001).

On M2 macrophages we see a similar pattern with hypoxia: it significantly upregulated *Arg1* expression in all the conditions. ADO, however, presented very heterogeneous effect; while it significantly upregulated the expression in ADO-normoxia compared to Control-normoxia, this effect was downregulated in hypoxia. Inhibitors, on the other hand, seemed to globally increase the expression in normoxia but slightly reduce it in hypoxia.

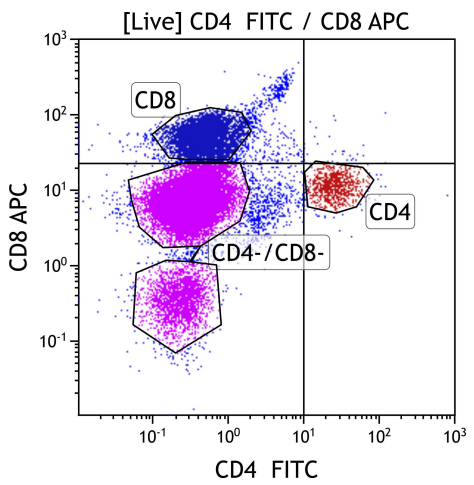
Finally, it is important to note that even though M1 macrophages expression was very low (***Fig.23***), the differences were statistically significant, they may however not be functionally different. The data still give important informations about the effects of hypoxia and ADO on the dynamic M1/M2 state.

4.3 Adenosine and hypoxia effects on T cell reactivation

4.3.1 Assessment of OT-1 phenotype

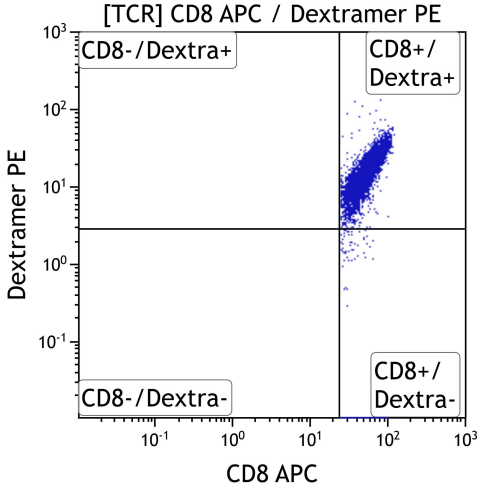
OT-1 mice were phenotyped in order to confirm the presence of the transgene. Two mice were randomly chosen and sacrificed. We took out their spleens in order to test their T cells TCR specificity for OVA with Dextramers (***Fig.24***). Dextramers are staining technology tools designed to obtain sharp results and detect very low concentration of antigens. They are composed of a backbone chain of dextran linked with fluorophores and many MHC-peptides complexes, MHC-OVA in our case. This allows capture and detection of low affinity T cells and in our case confirms the Ovalbumine specificity of our OT-I TCR.

A.



Gate	Number	%Total	%Gated
All	46 720	60,74	100,00
CD4	827	1,08	1,77
CD4-/CD8-	37 424	48,65	80,10
CD8	6 682	8,69	14,30

B.



Gate	Number	%Total	%Gated
All	6 650	8,65	100,00
CD8-/Dextra-	0	0,00	0,00
CD8-/Dextra+	0	0,00	0,00
CD8+/Dextra-	854	1,11	12,84
CD8+/Dextra+	5 796	7,54	87,16

Figure 24: OT-I Phenotyping quality control. *Splenocytes were stained for Live/Dead, CD4, CD8, CD19, TCR, TCR (OVA-specific, see* ***table 2****). **A.** Cells were gated on singulets then live cells. We see that lymphocytes represent a proportion of 16.07% of the overall cells. **B.** Cells were then gated on TCR positive-cells to check CD8 versus Dextramer. Majority of the $CD8^+$ were also positive for Dextramer, assessing the quality of our mice strand for the upcoming experiments.*

4.3.2 Extracellular ADO modifies the expression of surface activation and exhaustion markers on CD8 T cells

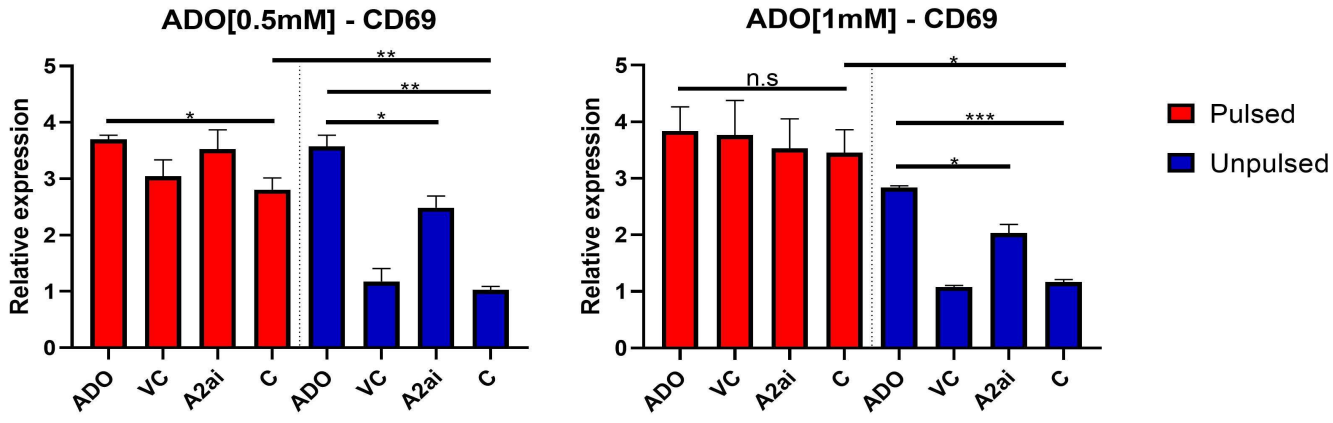


Figure 25: ADO upregulates CD69 expression in unreactivated CD8. *ADO: Adenosine; VC: Vehicle control (A2ai only); A2ai: ADO + A2ai; C: Control (Untreated cells). Splenocytes were extracted from OT-I mice and stimulated with OVA before being incubated. After a week of culture, half of the cells were mixed with macrophages presenting OVA (pulsed), the other half were mixed with macrophages not presenting OVA (unpulsed). Cells were then treated (0.5 or 1 mM ADO and/or A2ai), and incubated for three days at 21% O_2. The present results are issued from flow cytometry analysis, using immunofluorescent surface antibodies (anti-CD69) where cells were gated on singulet, live, $CD8^+$ cells. The results are the relative expression divided isotype. Statistical analysis was done using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p*≤0.01; *** p≤0.001.*

The activation marker CD69 is effectively and significantly overexpressed by the reactivated control cells when compared to the unreactivated control cells (***Fig.25***). Interestingly, ADO upregulates CD69 expression in both pulsed and unpulsed cells. Interestingly, the increase is stronger in the unpulsed group to the point that the expression of unpulsed control cells (0.5 mM) in on part with that of pulsed ADO cells (0.5 mM).

Both 0.5 mM and 1 mM ADO concentrations show a similar pattern where ADO itself seems to be sufficient to upregulate CD69 expression compared to control. A2ai partially restores CD69 expression in unpulsed cells, however pulsed cells seem to not respond to the treatment. Note that none of the vehicle controls presented a significant difference compared to their respective controls.

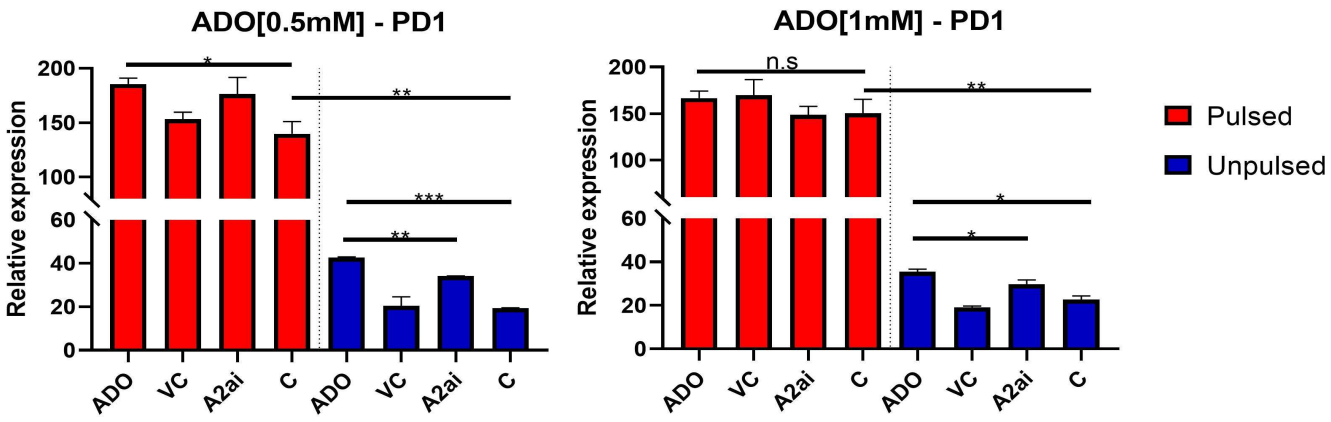


Figure 26: ADO upregulates PD1 expression in unreactivated CD8. *ADO: Adenosine; VC: Vehicle control (A2ai only); A2ai: ADO + A2ai; C: Control (Untreated cells). Splenocytes were extracted from OT-I mice and stimulated with OVA before being incubated. After a week of culture, half of the cells were mixed with macrophages presenting OVA (pulsed), the other half were mixed with macrophages not presenting OVA (unpulsed). Cells were then treated (0.5 or 1 mM ADO and/or A2ai), and incubated for three days at 21% O_2. The present results are issued from flow cytometry analysis, using immunofluorescent surface antibodies (anti-PD1) where cells were gated on singulet, live, $CD8^+$ cells. The results are the relative expression divided isotype. Statistical analysis was done using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p*≤0.01; *** p≤0.001.*

The exhaustion marker PD1 presents a large overexpression in pulsed compared to unpulsed cells for all the conditions, confirming the implication of reactivation in PD1 expression (***Fig. 26***). Similarly with CD69 results (***Fig.25***), ADO is pushing this overexpression a bit farther compared to control at both concentrations, in pulsed and unpulsed cells, however the delta is more important on unpulsed cells.

It appears that A2ai in unpulsed but not pulsed cells, is slightly but significantly counterbalancing the PD1 overexpression induced by ADO. Note that none of the vehicle controls presented a significant difference compared to their respective controls.

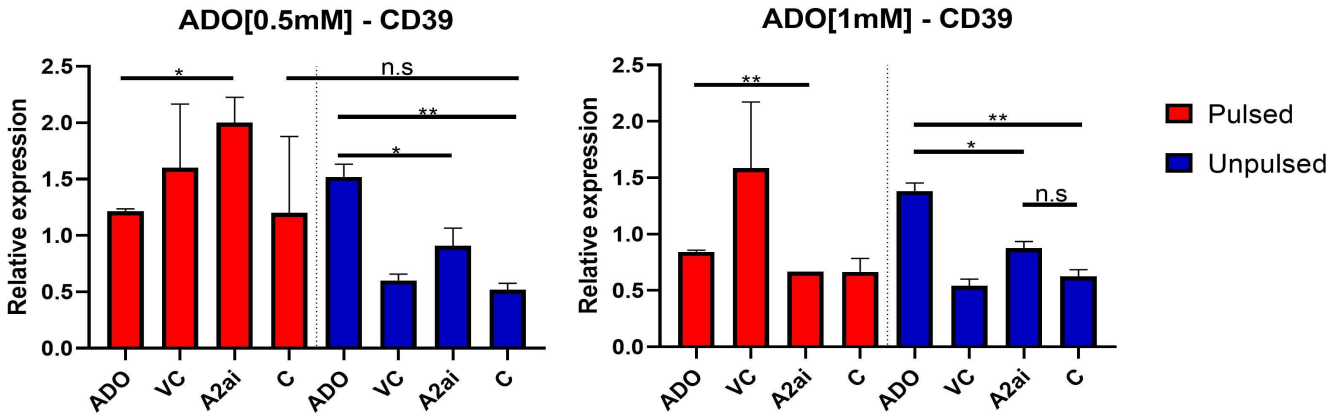


Figure 27: ADO upregulates CD39 expression in unreactivated CD8. *ADO: Adenosine; VC: Vehicle control (A2ai only); A2ai: ADO + A2ai; C: Control (Untreated cells). Splenocytes were extracted from OT-I mice and stimulated with OVA before being incubated. After a week of culture, half of the cells were mixed with macrophages presenting OVA (pulsed), the other half were mixed with macrophages not presenting OVA (unpulsed). Cells were then treated (0.5 or 1 mM ADO and/or A2ai), and incubated for three days at 21% O_2. The present results are issued from flow cytometry analysis, using immunofluorescent surface antibodies (anti-CD39) where cells were gated on singulet, live, $CD8^+$ cells. The results are the relative expression divided isotype. Statistical analysis was done using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p*≤0.01; *** p≤0.001.*

CD39 is another valuable exhaustion marker when it comes to CD8 T cells. The results on pulsed cells were however very heterogeneous, we will therefore only consider unpulsed cells results.

First of all and in contrast to PD1 results, it seems that CD39 is not overexpressed in pulsed versus unpulsed cells (***Fig.27***). Furthermore, both ADO concentrations triggered a clear CD39 overexpression (unpulsed) that was partially but significantly counterbalanced by the addition of A2ai. Note that vehicle control did not present a significant difference in CD39 expression in comparison to control.

4.3.3 Effects of ADO on intracellular cAMP concentration on Jurkat cells and OT-I CD8

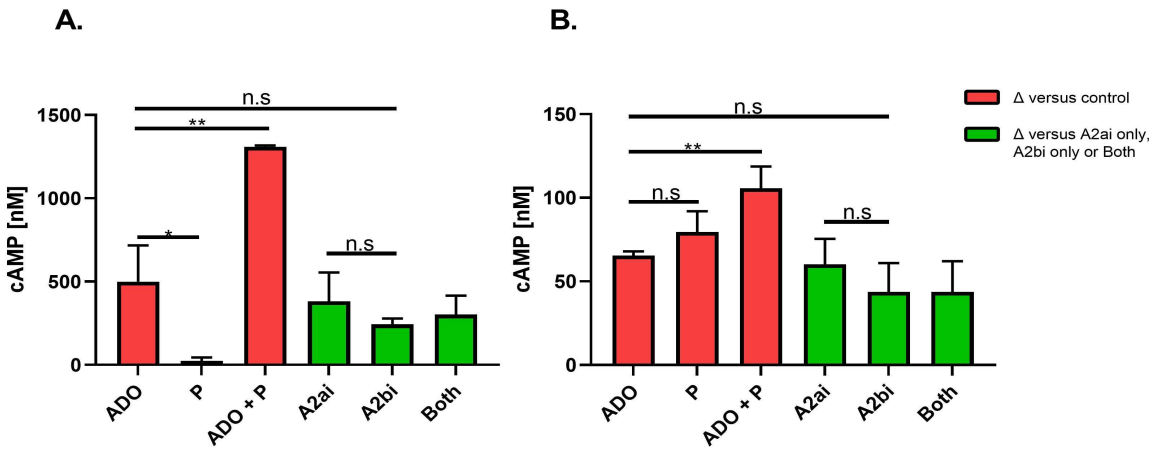


Figure 28: ADO in combination with Pentostatin effectively upregulates cAMP generation. *ADO: Adenosine; P: Pentostatin; ADO + P: Adenosine + Pentostatin; A2ai: Adenosine + A2ai; A2bi: Adenosine + A2bi; Both: Adenosine + A2ai + A2bi.* ***A.*** *Jurkat cells were thawed, cultured for one week then passed. Cells were treated after a first passage and incubated at 21% O_2 for 3 days whereafter their media was taken for this analysis.* ***B.*** *Splenocytes were extracted from OT-I mice and stimulated with OVA before being incubated. After a week of culture, cells were reactivated with Dynabeads^{tm} (Thermofisher, 1:1 concentration) and treated for three days at 21% O_2. Their media was finally taken in order to perform this analysis. All the present results are issued from cAMP assay and represent the delta versus untreated cells (red) or versus vehicle control (inhibitors, green). Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p≤*0.01.*

cAMP generation was monitored on human T cell cancer cell line (Jurkat) and in murine CD8 (reactivated OT-I) in order to have a more direct quantitative overview of the effect of our inhibitors on ADO since extracellular ADO induce cAMP generation. Similarly with previous results (***Fig.17***), cAMP generation was increased in every condition compared to control and vehicle control except with Pentostatin-only (***Fig.28A***). On the other hand, ADO + P induced a significantly higher cAMP generation compared to ADO-only, which was the effect we expected. It confirmed the importance of ADA on the clearance process of extracellular ADO.

In both models (**A**, **B**), cAMP concentration seemed to be slightly reduced by the inhibitors, especially A2bi, however none of the reductions was significant. Surprisingly, on the OT-I CD8, Pentostatin-only increased cAMP concentration in a similar order of magnitude to that of ADO-only (***Fig.28B***), which was opposite to what we observed on Jurkat and Raw models (***Fig.28A, Fig.17***). The combination (ADO + P) again importantly increased the concentration compared to ADO-only on the OT-I model. Finally, we can note that the overall concentration of cAMP was ten times higher in the human cancer cell model (***Fig.28A***) compared to the murine healthy cells (***Fig.28B***).

4.3.4 Effects of extracellular ADO on OT-I CD8 IFN-γ secretion

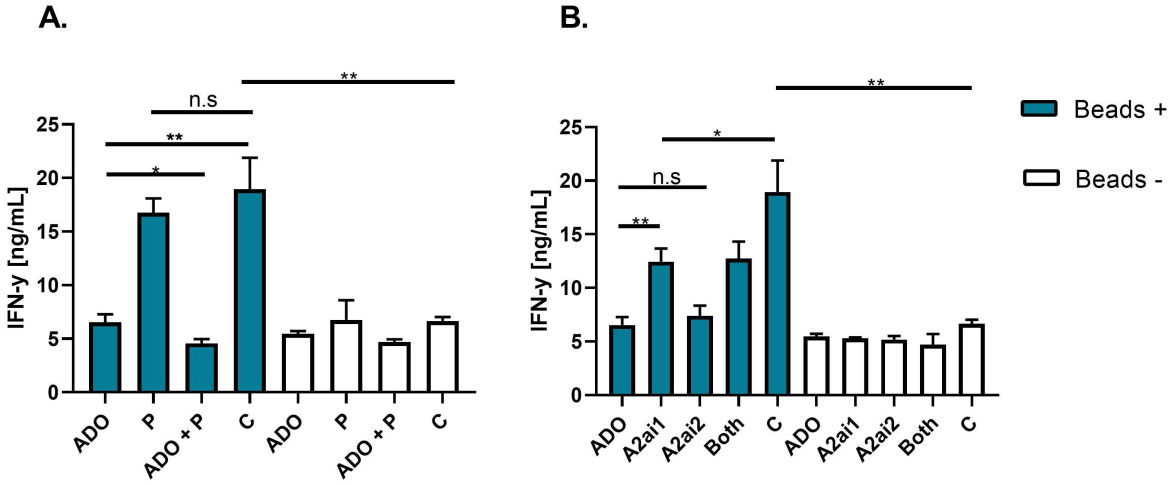


Figure 29: Pentostatin in combination with ADO lower IFN-γ secretion but Istradefylline partially restores it. *ADO: Adenosine; P: Pentostatin; ADO + P: Adenosine + Pentostatin; A2ai1: Adenosine + A2ai1 (Istradefylline); A2ai2: Adenosine + A2ai2 (Cpi-444); Both: Adenosine + A2ai1 + A2ai2; C: Control (Untreated cells). Splenocytes were extracted from OT-I mice and stimulated with OVA before being incubated. After a week of culture, half of the cells were reactivated with Dynabeads^{tm} (Thermofisher, 1:1 concentration) the other half remained unreactivated. All were treated for three days at 21% O_2. Their media was finally taken in order to perform this analysis. The present results are issued from ELISA kits (BD OptEIA^{TM}). Statistical significance was determined using GraphPad Prism paired parametric T test; * p≤* 0.05 ; ** p≤*0.01.*

Our interest was directed to the effect of extracellular ADO in CD8 T cells effector functions. We decided to measure the CD8 T cells IFN-γ secretion in order to answer this question. In the first panel (***Fig.29A***), Pentostatin was used in order to figure out if the inhibition of ADA alone was enough to downregulate IFN-γ secretion otherwise, if the combination of ADO + P was effecting this secretion more than ADO alone. The latter, therefore confirming the role of ADA in the ADO clearance and its importance as a potential target in further experiments. In the second panel, we tested a new A2a inhibitor (A2ai2, *Cpi-444*) and compared its effect with the one that was used for all the other experiments (***Fig.29B***).

A clear higher secretion was observed from the reactivated cells group compared to the unreactivated one when comparing Control groups. This was observed in both panels. A significant reduction of IFN-γ secretion is also observed in both panels when comparing ADO groups with Control groups. Pentostatin alone is not reducing IFN-γ secretion, however when combined with ADO, the condition presented the lowest IFN-γ concentration. It was significantly lower than the ADO-only condition, therefore confirming our hypothesis (***Fig.29A***). The group treated with A2ai1 presented a partial but significant recovery of IFN-γ secretion, while the other inhibitor did not improved the secretion compared to ADO condition (***Fig.29B***).

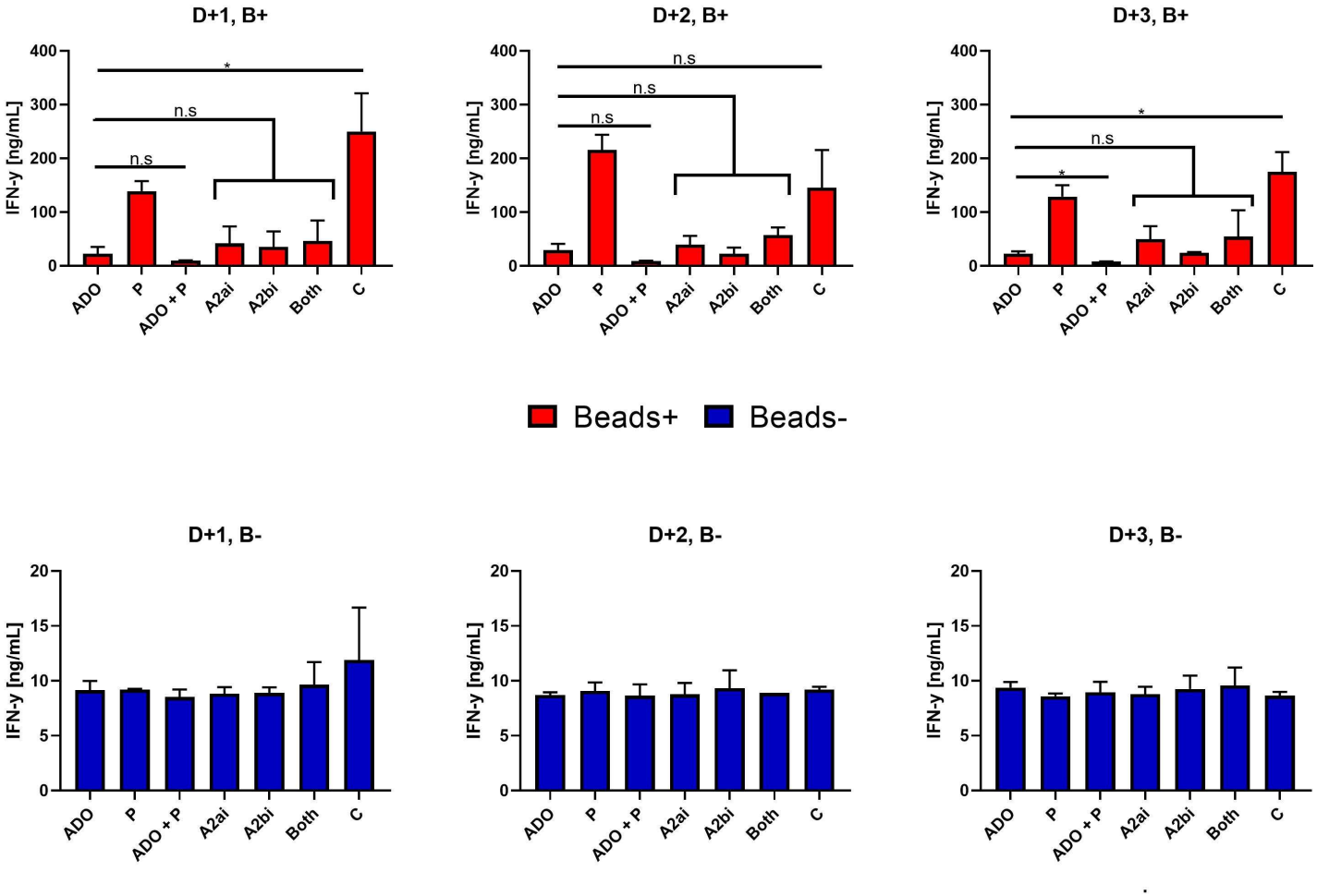


Figure 30: A2ar inhibition partially restores IFN-γ secretion. *ADO: Adenosine; P: Pentostatin; ADO + P: Adenosine + Pentostatin; A2ai: Adenosine + A2ai; A2bi: Adenosine + A2bi; Both: Adenosine + A2ai + A2bi; C: Control (Untreated cells). Splenocytes were extracted from OT-I mice and stimulated with OVA before being incubated. After a week of culture, half of the cells were reactivated with Dynabeads^{tm} (Thermofisher, 1:1 concentration) the other half remained unreactivated. All were treated for a maximum of three days at 21% O_2. Their media was taken 24h/48h/72h post treatment, in order to perform this analysis. The present results are issued from ELISA kits (BD OptEIA^{TM}). Three biological replicates were used to complete this experiment. Statistical significance was determined using GraphPad Prism paired parametric T test; * $p \leq 0.05$.*

Lastly, our interest was turned toward the kinetics of the compounds we are using as well as the cell response to ADO over time. It was decided to measure their IFN-γ secretion at different time points to a maximum of three days in order to stay consistent with the previous experiments.

First of all we see that unreactivated cells do not present a variation of secretion over time nor between conditions (***Fig.30***), the order of magnitude is similar to that of our previous results (***Fig.29***). On the other hand we see that ADO already has an impact of IFN-γ secretion first day post-treatment and is slightly reducing the secretion day after day. This effect might be due to cell death and/or lack of stimulation because the same effect was observed in control condition.

Pentostatin-only was one more time not reducing the secretion but also did again reduced IFN-γ to its lowest level when combined with ADO. This effect was visible at the three time points (D+1, D+2, D+3). Finally, the inhibitors did slightly restored IFN-γ secretion, especially A2ai at D+3, but none of the inhibitors results were significant compared to ADO this time, the variation measured between biological replicates is a probable reason to explain the lack of significance. The data still provide an interesting trend that is consistent with previous results and literature.

5 Discussion

We mentioned that hypoxia is a feature commonly found within solid tumours and that this oxygen deprivation is accompanied by several side effects which include extracellular ADO release. In this study we focused our interest on the effects of the extracellular ADO and hypoxia on the polarization mechanism of macrophages as well as its effects on T cell reactivation, two main actors of the antitumour immunity.

The provided data confirm that ADO is an important actor in antitumour immunity with a plethora of effects. Macrophage results however suggest a mostly beneficial effect of ADO in regards to the M1/M2 balance (***Fig.17***-***Fig.23***), which is not consistent with literature that says that ADO promotes alternative macrophage activation (M2) through A2br activation. More genes are therefore needed in order to clearly validate the dynamic state of the macrophages.[76,77]

While ADO and hypoxia were expected to downregulate M1 surface markers such as CD80 and CD86, flow cytometry results showed that these markers were mostly upregulated or unchanged both in M1 and M2 macrophages (***Fig.15***, ***Fig.16***). This was confirmed as well with qPCR data that showed that hypoxia and ADO were upregulating M1 expression markers (*Nos2*, *Stat1*, *Il-1b*) on M2 macrophages (***Fig.19***, ***Fig.20, Fig.21***), while downregulating two of the three M2 expression markers we used (*E-Cad*, *Mrc1;* ***Fig.22***, ***Fig.23***). It is important to note that these M2 macrophages were expressing more M1 markers than the real M1-control macrophages. The gene expression of M1 macrophages was very heterogeneous in response to the treatments but M1 markers were mostly downregulated or unchanged, while M2 markers were upregulated or unchanged by hypoxia but downregulated or unchanged by ADO.

Our results suggest that hypoxia and ADO may have a beneficial role in the M1/M2 balance, specifically on M2 macrophages to push them back toward a more M1-like phenotype. The underlying pathways need more validations, however some studies suggest that A2a receptor features a negative feedback loop that plays a role in the reduction of inflammation.[78,79] In regard to this theory, plus knowing that macrophages are usually cultured for shorter time than the 3 days post-treatment we have done, it is highly possible that our M2 macrophages started to turn M1 because of A2a negative feedback, overcoming the immunosuppression, thereby shifting their balance.

The cAMP results (***Fig. 17***) also match this hypothesis in a sense that cAMP generation is still very high after three days of treatment compared to control, meaning that the possible phenotype switch is not due to a direct diminution of ADO. Despite the model being different (cancer cell line), the theory remains interesting and further experiments are necessary in order to measure if such a negative feedback is possible *in vitro* in cancer cells.

Recent literature also states that an M2 to M1 macrophage phenotype switch is possible through TLR-3 activation,[80] plus we also know that there are different M2 macrophages subsets (M2a, M2b, M2c, M2d) with different expression profiles and functions.[81] It would be interesting in further experiments to measure TLR-3 expression on macrophage populations and try to determine if this receptor is somewhat linked with ADO pathway.

In the literature, CD8 T cells are described as exhausted in presence of high concentrations of extracellular ADO, an environment that is found in injured tissues and tumours because of the inflammation.[82,83] Our results are partially consistent with literature in a sense that ADO is effectively upregulating PD1 expression (***Fig.26***) but only on unreactivated cells. Reactivated CD8 cells, in comparison were expressing a lot of PD1 but this was due to the reactivation only.

Secretion assays were consistent with literature as we see that ADO alone was sufficient to drastically reduce IFN-γ secretion for the three days of the treatment (***Fig.30***) proving that the effect is persistent. cAMP results corroborate these results as well with high level of cAMP after three days as well (***Fig.28***). It is interesting to note that inhibiting ADA as well as adding ADO was exacerbating this effect, confirming the enzyme to be a potential interesting target in the ADO pathway. Recent literature with experiments done *in vitro* corroborate this proposition.[84]

Even thought inhibiting A2 receptors seemed to partially restore the T cell secretion of pro-inflammatory cytokines (IFN-γ; ***Fig. 29, Fig. 30***), promoting the ADO to inosine conversion might be a more potent way to counterbalance ADO immunosuppressive properties. Adenosine is an abundant molecule involved in many signalling pathways and with a plethora of roles, we have to be cautious when modifying the balance. That is why the specific inhibition of certain ADO receptors is interesting.

5.1 Concluding remarks

Overall, our results confirm high heterogeneity of different human GBM samples based on their expression profiles of genes associated with hypoxia and metabolism. We also confirmed that different glioma cell lines do not react the same way when confronted with hypoxia, again highlighting their heterogeneity.

Regarding macrophage polarisation, and contrary to what has been previously proposed, we observed a mostly beneficial (pro-M1) effect of ADO and hypoxia regarding the M1/M2 balance, at least based on the selection of genes for which we measured expression. Concerning T cells, our data confirmed that CD8 effector functions are impaired by high doses of extracellular ADO, however, this could be partly counterbalanced by using an A2ar inhibitor.

T cells and macrophages are major actors of antitumour immunity, therefore, finding treatments that restore and enhance their functions, even in the context of tumour cell and tumour microenvironment heterogeneity, is of top priority in order to improve patients' life expectancy and quality of care.

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